SOME PHYSICAL PARAMETERS AND INTERACTION PROPERTIES OF THE PROTEOGLYCAN AND RELATED GLYCOSAMINOGLYCANS OF BOVINE NASAL CARTILAGE

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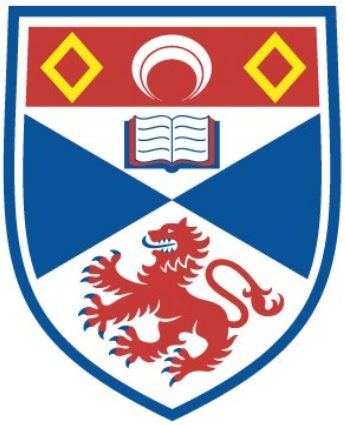
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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SOME PHYSICAL PARAMETERS AND INTERACTION PROPERTIES OF THE PROTEOGLYCAN AND RELATED GLYCOSAMINOGLYCANS OF BOVINE NASAL CARTILAGE

by

PETER J. WELLS

A thesis presented to the University of St. Andrews for the Degree of Doctor of Philosophy

Department of Biochemistry,
University of St. Andrews. 1971

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, St. Andrews, under the direction of Dr. A. Serafini-Fracassini.

C E R T I F I C A T E

I hereby certify that PETER J. WELLS has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1964, and graduated with the degree of Bachelor of Science , first class, in July 1968.

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INTRODUCTION.

In view of the comparative history of nucleic acid, protein and connective tissue biochemistry, the slow progress in the last mentioned field is, at first sight, surprising. Even by 1871, Kölliker had suggested that collagen and 'mucin' were secreted by the same cell type, and later, in 1876, Flemming observed that extraction with lime water caused tendon fibre bundles to split up into very fine fibrils, thus indicating the possible existence of a fibre stabilising factor. A few years later, the fact that chondroitin sulphate could only be extracted from cartilage in good yield with strongly alkaline solutions, was recognised by Mörner (1889) and others as an indication that the polysaccharide was attached to protein.

However, in spite of this early start, paralleled in other fields of biochemistry, it was not until the late 1950s (Mathews, 1956; Webber and Bayley, 1956; Malawista and Schubert, 1958; Mathews and Lozaityte, 1958) that the so-called mucin was recognised as a distinct macromolecular species. Even now, although the chemistry of collagen, the major connective tissue protein, is fairly well understood, the precise molecular organisation of the proteoglycans is still largely speculative. An early problem was the great difficulty in solubilizing a substance in such

close association with collagen. Later, the discovery of a considerable degree of heterogeneity among the proteoglycans was another set-back to structural studies. A further problem arises from their polyelectrolyte nature. The theoretical complications which have to be introduced into the physical chemical characterisation of polyions are frequently difficult to apply practically, and in many cases (though primarily in the thermodynamics of ternary systems) the theoretical background has only just been developed by the physical chemists.

A more specific problem in the study of proteoglycans lies in the definition of the basic macromolecular unit. Generally its molecular weight has been suggested to be of the order 1 - 5 million. (Bernardi, 1957; Mathews and Lozaityte, 1958; Luscombe and Phelps, 1967). More recently, Buddecke et al.,(1963),Meyer,(1966) and Partridge (1966), have each presented evidence of different units with molecular weights ranging down to 240,000, while Rosenberg et al. (1970), following on from the work of Sajdera and Hascall (1969) have proposed a sub unit of molecular weight 2.4 million, which can, together with a linkage glycoprotein, form what the authors term 1st and 2nd order aggregates, with diameters in the range 5,000 - 8,000 Å. The evidence for these is based on electron microscopy on in vitro preparations. There appears

to be no evidence as yet that these aggregates exist in vivo and it may well be that these are merely 'in vitro phenomena'.

An alternative approach to the problem of the basic unit has been suggested by the work of Serafini-Fracassini et al. (1971a ; 1971b). From work on chemically split PG the authors have identified two units of 33,000 and 120,000 molecular weight which they have subsequently cited as intermediates in PG biosynthesis. Whether or not these subunits are ultimately confirmed in vivo, if they are shown to be homogeneous, then there exists the possibility of getting detailed structural information, hitherto unobtainable, from analysis of the 1 - 5 million molecular weight species. It is to this end that the work described in the second part of this thesis is directed. It is intended as a pilot study

a) to examine the feasibility of ultrafiltration methods in fractionation

and

b) to investigate the applicability and limitations of certain physical methods of analysis.

The interaction studies presented in part 1 arose out of interest in the possible in vivo significance of low molecular weight PG species. Again, as in the case of the organisation of PG, there is still little solid fact regarding the in vivo relationship between collagen and mucopolysaccharide. The first studies on collagen/glycosaminoglycan interactions were

carried out using insoluble collagen by Einbinder and Schubert (1951) who concluded that

1. Only anions were fixed.
2. Maximum fixation was at pH 3.7.
3. Fixation was equivalent to the number of cationic groups.
4. There was no interaction at neutral pH.

In fact Mathews (1965) was the first to demonstrate that collagen binds with mucopolysaccharide under conditions close to those in vivo. On performing free electrophoresis with soluble collagen (actually a solubilized polymeric collagen of very high molecular weight) plus chondroitin sulphate, hyluronate or heparin, he noticed an extra peak which he interpreted as being due to an interaction product. In his discussion Mathews made a number of conclusions which in many ways summarize the present state of knowledge on the subject.

1. Interaction requires the undenatured state of collagen.
2. Interaction is reversible and mainly due to electrostatic forces
3. Interaction depends upon both linear charge density and chain length of the polyanion.
4. Interaction might involve to some extent molecular entanglement and excluded volume effects.

Since then the suggestions regarding electrostatic forces have been basically confirmed. (Steven et al.,1968; Wasteson and Obrink, 1968; Obrink, 1969; Steven et al., 1969). Almost all these studies have been carried out either with tropocollagen or with a gel form produced in vitro (Obrink and Wasteston, 1971) and if these studies are intended to be possible models of in vivo processes then it should be established that soluble collagen is in fact present in cartilage in sufficient amounts to take part in such interactions. This is an important point since it seems entirely reasonable that collagen (containing basic groups) should react with <u>any</u> anionic molecule such as the glycosaminoglycans or even DNA and that this binding should increase with decreasing pH and ionic strength. As it is, to date, there do not appear to have been any successful attempts to obtain soluble collagen from cartilage and in the light of this it would seem that investigations of interactions in soluble systems are of doubtful value. As yet the difficulties of working with insoluble systems put a limit to accurate representaion of in vivo interactions. However, in view of the considerable variability in the inter-relationship between different proteoglycans and collagen in connective tissue matrices which are reported by various authors (Serafini-Fracassini and Smith, 1966; Smith and

Serafini-Fracassini, 1968; Smith and Frame, 1969) any in vitro experiments will only give a general picture of the in vivo interactions.

The experiments described in this thesis were intended to provide a comparative picture of the uptake of three dissimilar mucopolysaccharides. The first - heparin, as an example of a low molecular weight highly discharged polyanion The second - chondroitin sulphate, as a medium molecular weight (50,000) component of the third substance - proteoglycan - the macromolecular species which appears to be the form in which almost all glycosaminoglycans appear in vivo. In this case, the proteochondroitin-4-sulphate. Advantage was taken of the bismuth nitrate staining procedure to provide information as to the disposition of the mucopolysaccharide and collagen in the experimentally formed complex.

PART ONE

MATERIALS AND METHODS

Extraction of fibrillar collagen.

Fresh ox achilles tendon was washed with water and then 1 M NaCl. The fibres were then teased apart and extracted with several changes of 1 M NaCl for 72 hours at 4°C. After washing for 24 hours with distilled water the fibres were re-extracted with ethanol/chloroform (1:3), washed with ethanol and dried in a desiccator. The material was finally milled to a fibrous powder.

Assessment of glycosaminoglycan uptake.

To assess the role of electrostatic forces in such interactions, an attempt was first made to determine the number of cationic groups available on collagen to react with anionic molecules. For this purpose 1-naphthol-4-sulphonic acid was chosen as an anionic dye for the following reasons.

a) It has a high extinction coefficient in a convenient part of the U.V. spectrum (295nm).

b) Its small size (compared to the more commonly used dyes with absorption maxima in the visible region) increases the probability of stoichiometric binding.

To test this, the uptake of the dye by gelatin in which all the cationic groups are necessarily available was measured. The gelatin was derived by exhaustive autoclaving of the ox tendon collagen used in the subsequent experiments. Before measuring the dye uptake, the gelatin was removed from solution by prolonged high speed centrifugation.

Since the possibility existed that hydrogen bond formationbetween successive dye molcules could give misleading uptake values, experiments were carried out using 2-naphthol-6-sulphonic acid and naphthalene-1-sulphonic acid. However the results were within the range of values obtained for 1-naphthol-4-sulphonic acid.

Determination of the uptake of both dye and glycosaminoglycan by the fibrillar collagen was carried out as follows:- 30 mg of collagen were suspended in 25 ml of 0.01 M KCl containing a fixed amount of 1-naphthol-4-sulphonic acid. The pH was adjusted and after an equilibration period of at least 3 hours (uptake values stabilized after about 2 hours) dye uptake was determined and 40mg of polysaccharide added in 5.0 ml of 0.01 M KCl. Chondroitin sulphate was obtained from Sigma; lot no 15B - 0010; heparin from Wilson

Laboratories, Chicago, Illinois; lot no 136651. The PG was extracted as described in part 2 of this thesis. The system was then left to equilibrate for a further hour and after removal of collagen by centrifugation, the concentration of glycosaminoglycan in the supernatant was determined by both differential refractometry and hexuronic acid estimation. (Bitter and Muir, 1962). The pH was kept constant within 0.2 units during the whole experiment. Concentration of dye was estimated by reference to a standard graph of absorbance plotted against concentration, the slope of which was found by the method of least squares. Visual inspection of the graph (Figure 1) confirmed that Beer's law was apparently obeyed for the range of concentrations used in the experiments and further checks showed that the extinction of the dye was unaffected in the pH range covered by the uptake experiments.

The first series of experiments were carried out in especially constructed vessels which had a low surface area to volume ratio and at the same time allowed addition or removal of material and continuous monitoring of pH. The solution was stirred magnetically. The subsequent acquisition of a Radiometer Titrator allowed more precise control of pH (better than $\pm$ 0.2 pH unit) but the built-in stirrer

rotated approximately 10 times faster than the flea used in the previous experiments. Later it was found that the speed of stirring affected the uptake of polysaccharide and for this reason the first series of experiments are designated 'low speed' and the second 'high speed'.

Electron Microscopy.

Bismuth nitrate staining was used to visualize the inter-relationship of polysaccharide chain and collagen fibril. Bismuth nitrate does not stain protein (Albersheim and Killias, 1963) but the ions bind to one or more sulphate groups on the polysaccharide chain and cause neutralization of the net charge and cross linking of adjacent sulphates. The pK of the carboxyl group is about 3.4 (Mathews, 1961) so at a pH around 1.0 the group will be insufficiently ionized to allow interaction with the bismuth ion.

The bismuth nitrate stain was prepared by dissolving 1.0 g of bismuth nitrate in 10 ml of 2M nitric acid and then making up to 200 ml with distilled water giving a 0.5% solution in 0.1M nitric acid.

The collagen/glycosaminoglycan complexes produced at

specific pH values were sprayed on to carbon filmed grids and dried. The grids were then stained for 15 minutes, washed successively with 2M nitric acid and water and finally dried. (Serafini-Fracassini et al., 1969).

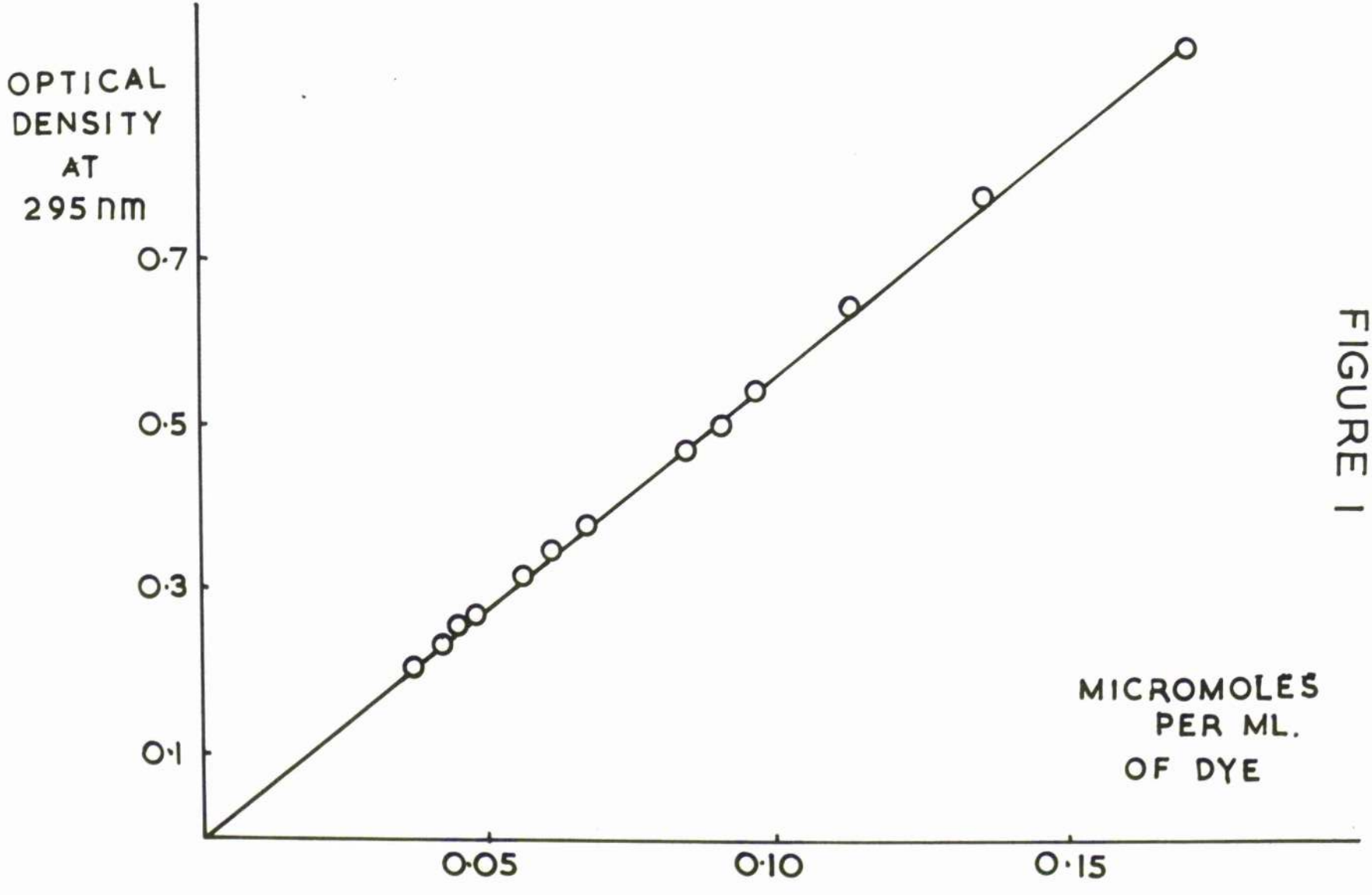

OPTICAL
DENSITY
AT
295 nm
0·7
0·5
0·3
0·1
0·05
0·10
0·15
MICROMOLES
PER ML.
OF DYE

FIGURE 1

RESULTS AND DISCUSSION

The gelatin derived from the collagen used in these experiments was found to have an uptake in the order 1 mM g^{-1}, a value which is in keeping with the number of basic groups in the protein. (Bowes and Kenten, 1948, estimated 0.90 mM g^{-1} from titration data and 0.94 mM g^{-1} from amino acid analysis). Results for uptake on collagen are shown in Table 1 (low speed) and Figures 2 (low speed; results expressed in µM per mg) and 3. Five estimations were carried for each value.

From Table 1 it is evident that the uptake of 1-naphthol-4-sulphonic acid by fibrillar collagen is in the order 1% of the value obtained for gelatin. This would suggest that the dye is bound only to those cationic groups available on the surface of the collagen fibrils. On a different level this is analogous with results obtained by Hartman and Bakerman (1966) who showed that native acid-soluble collagen has twice as many ionizable groups unavailable to titration as salt-soluble collagen. The slightly reduced uptake of the dye at higher pH values is most probably due to dissociation of dye from imidazole and α-amino groups (pK estimated by Bowes and Kenten to be in the region 7.5).

Table 1.

Glycosaminoglycan and proteoglycan (PG) uptake (mg/mg)

Heparin	0.426 ±.016	0.374 ±.003	0.344 ±.009	000
Chondroitin sulphate	0.397 ±.023	0.474 ±.011	0.492 ±.001	0.137 ±.007
PG	0.183 ±.011	0.273 ±.019	000	000

Dye uptake (μmoles/g)

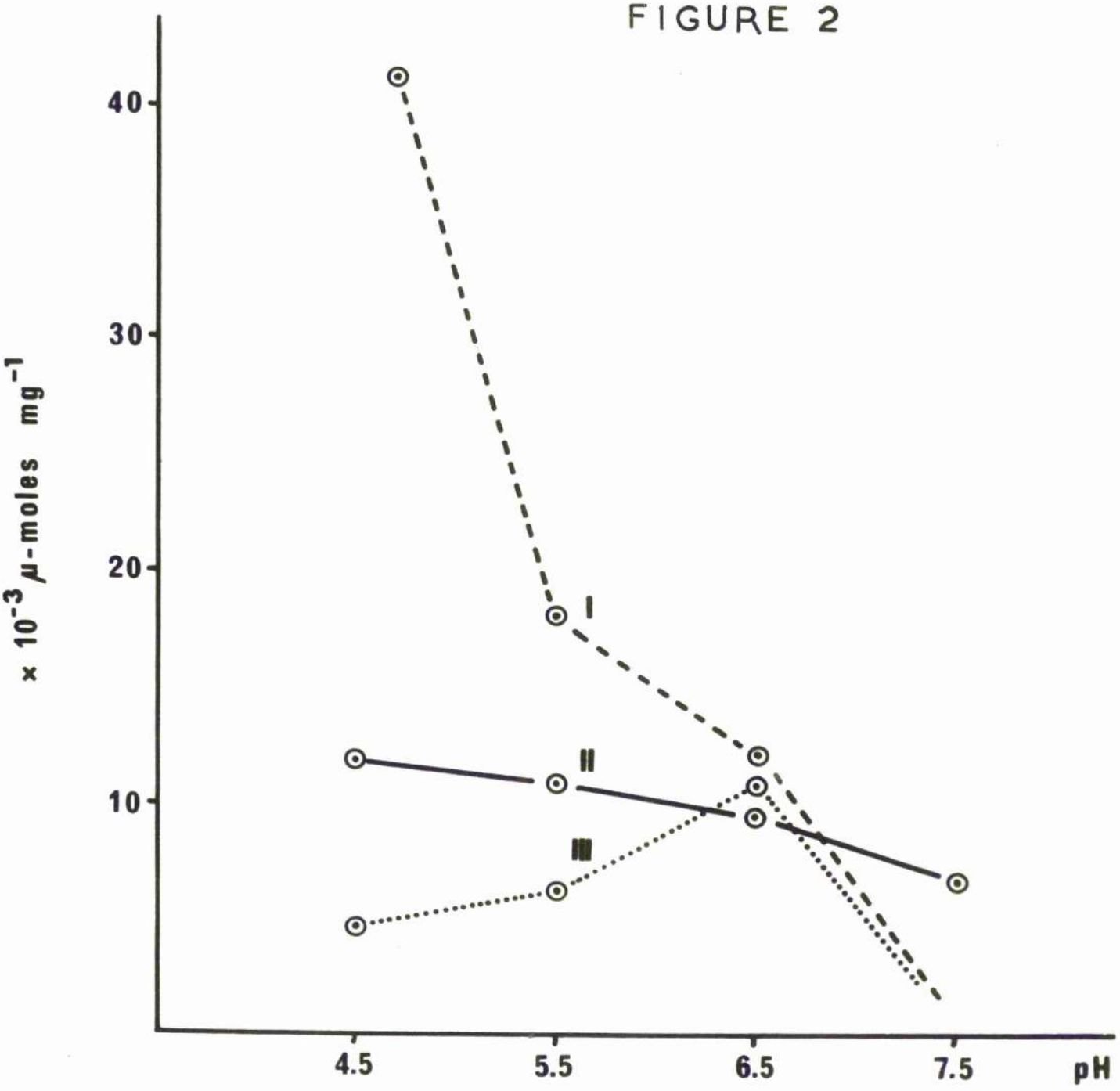
1-naphthol -4sulphonic acid	12.4 ±1.6	10.7 ±.9	10.4 ±.8	5.7 ±.7

The four columns show the uptake values at pH 4.5 ; 5.5 ; 6.5 ; 7.5 respectively, from left to right.

Figure 2

Uptake of polysaccharides and dye as a function of pH. The uptakes are expressed in μ-moles per mg of collagen.

FIGURE 2



I Heparin

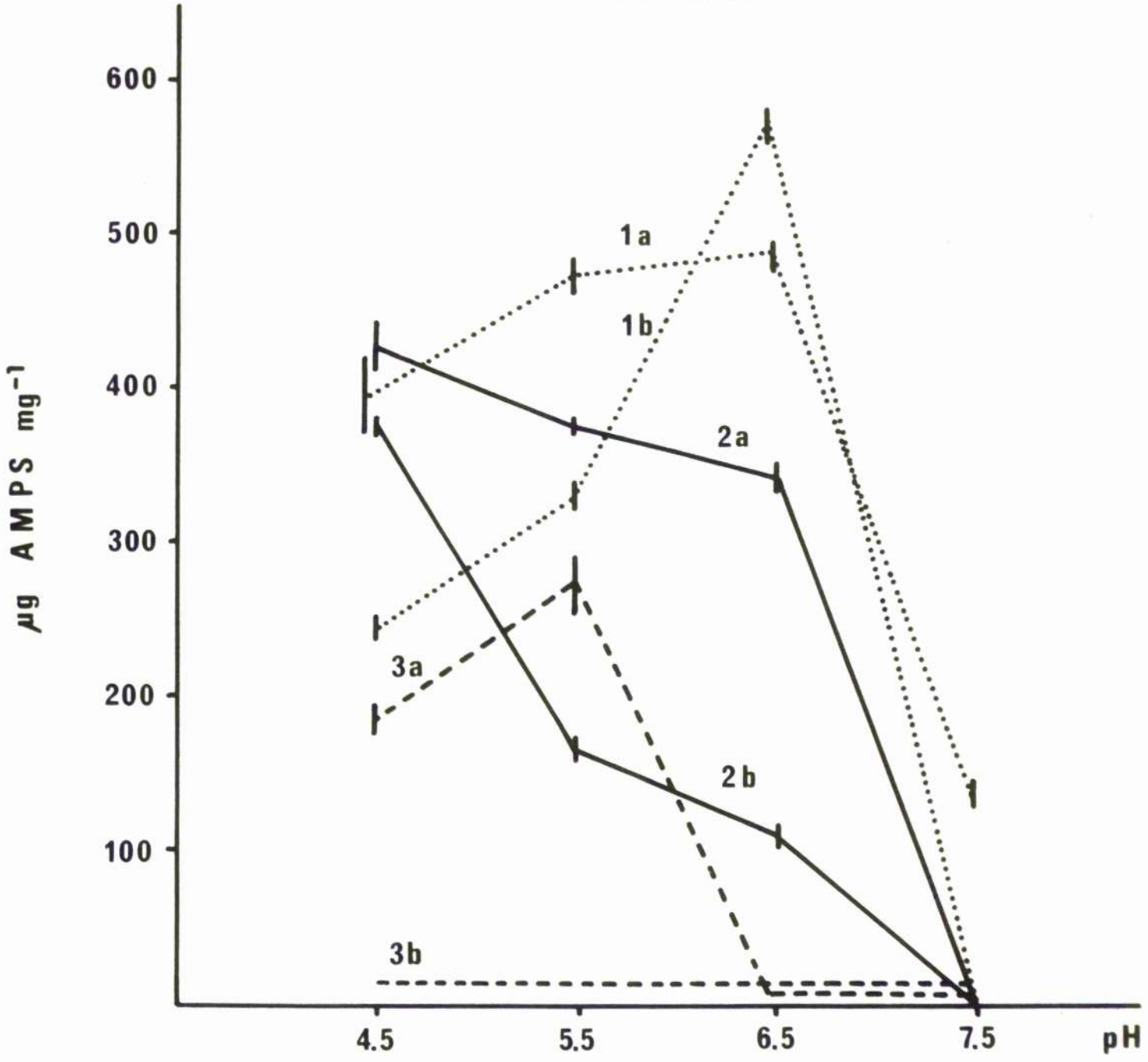
II 1-naphthol-4-sulphonic acid

III Chondroitin sulphate

Figure 3

Uptake of polysaccharides and PG as a function of pH with both high and low speed stirring. Uptakes are expressed in µg of acidic mucopolysaccharides per mg of collagen.

FIGURE 3



1 Chondroitin sulphate

2 Heparin

3 PP – L

a : low speed stirring

b : high speed stirring

The high uptake of heparin at low pH is to be expected from a molecule containing in the order seven ionizing groups per tetrasaccharide repeating unit. Predictably the uptake falls off with increasing pH while faster stirring (Figure 3) accentuates this. Heparin is a very minor component of connective tissue and almost certainly plays no structural role in cartilage. It was used in these studies hopefully to provide a norm to which the behaviour of chondroitin sulphate and PG could be compared, and its uptake pattern may reasonably be interpreted in terms of simple electrostatic interaction with basic groups in the protein.

In contrast to these results the binding of chondroitin sulphate, while comparable with that of heparin at low pH, reaches a maximum at pH 6.5 and is still considerable even at pH 7.5. It is noteworthy that the high speed stirring which drastically reduces the uptake of heparin at pH 5.5 and above, does not affect the maximal binding of chondroitin sulphate. With PG, although maximum binding occurs at pH 5.5 already by pH 6.5 the uptake is zero and in contrast to chondroitin sulphate, high speed stirring abolishes binding at any pH.

A number average molecular weight determination carried

out by osmometry on the chrondroitin sulphate sample gave a molecular weight of 53,000. $\overline{M}n$ by ultracentrifugation for heparin was 9,000. For PG the repeating "doublet" was assumed to be 40,000 (Mathews. 1958; Partridge, 1966).

If these molecular weights are taken into account (Figure 2) then it can be concluded that there is approximately one binding site on the collagen per glycosaminoglycan unit, which would imply a very weak interaction, at least as detected under the present experimental conditions. However it seemed likely that a certain proportion of macromolecules could be removed from solution by entanglement with others already bound to the surface of the collagen. To investigate this possibility, a further check was made on the amount of bound dye displaced by chondroitin sulphate during the experiment at low pH. Chondroitin sulphate was found to be suitable by virtue of its negligible absorption at 295nm. At pH 4.5, $6.5 \cdot 10^{-3}$ µ-moles of dye were released per mg of collagen by $7.5 \cdot 10^{-3}$ µ-moles of chondroitin sulphate. In this determination then, molecular entanglement is affecting the uptake by some 15% of the total value.

The results indicating a lack of interaction of PG with collagen at physiological pH are in agreement with other reports in the literature. For instance, Schubert (1966) reported that 'there is no evidence that PG can be

absorbed to collagen fibres if they are immersed in a solution of PG'.

These results are surprising, however, for the following reasons:

1. There is an intimate connection between PG and collagen in vivo as evident for example by electron microscopy and in the experimental difficulty of freeing PG from 'hydroxy proline containing material'.

2. Adult cartilage consists almost entirely of the insoluble form of collagen (Schubert, 1966) and for this reason primary interaction with soluble collagen is improbable, particularly so because it appears that newly synthesized collagen is not linked to proteoglycan. (Levene et al., 1966).

In addition to these problems, Maroudas (1970), in a study of the diffusion of solutes in articular cartilage, has concluded that , owing to steric exclusion, the largest molecules which can penetrate into cartilage are of the size of the haemoglobin molecule. Although this work is open to some criticism on the grounds that the experimental values she obtained are average values for several millimetres of cartilage and therefore may not reflect the situation at a molecular level, it does suggest difficulties in understanding the diffusion of PG within the cartilage.

Yet again Revel (1970), with autoradiographic studies

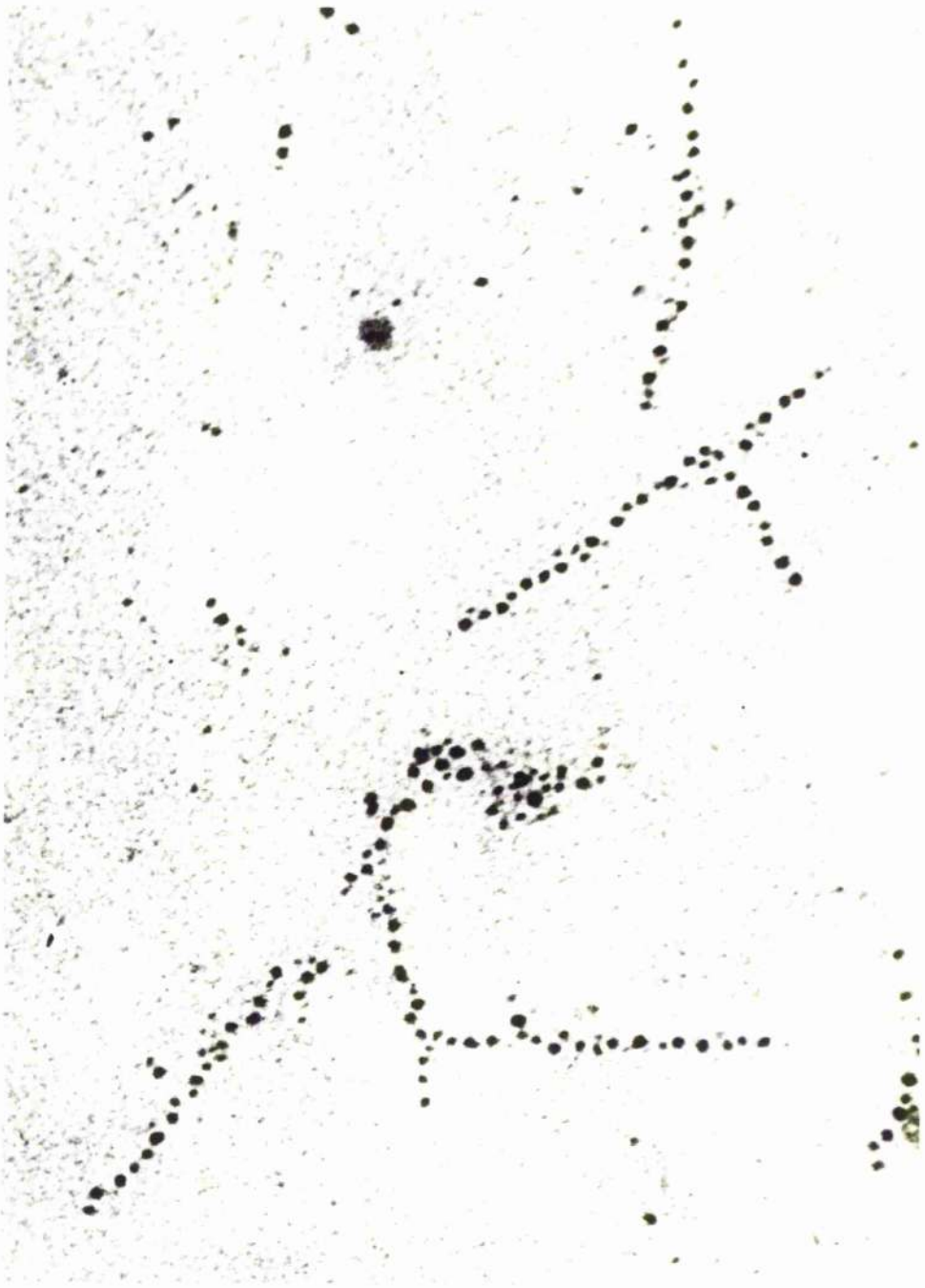
using labelled glucose has shown that after two hours, secreted material (assumed to be proteoglycan) is already several microns away from the cells.

In view of these difficulties then, it seems reasonable to suppose that if PG were liberated from the chondrocyte as a smaller unit, both diffusion and interaction at physiological pH would be possible. Support for this hypothesis has been obtained recently by Serafini-Fracassini et al. (1971).

A possible interpretation of the peak uptakes obtained for PG and chondroitin sulphate is suggested in the light of the electron microscopic observations on the collagen/glycosaminoglycan complexes.

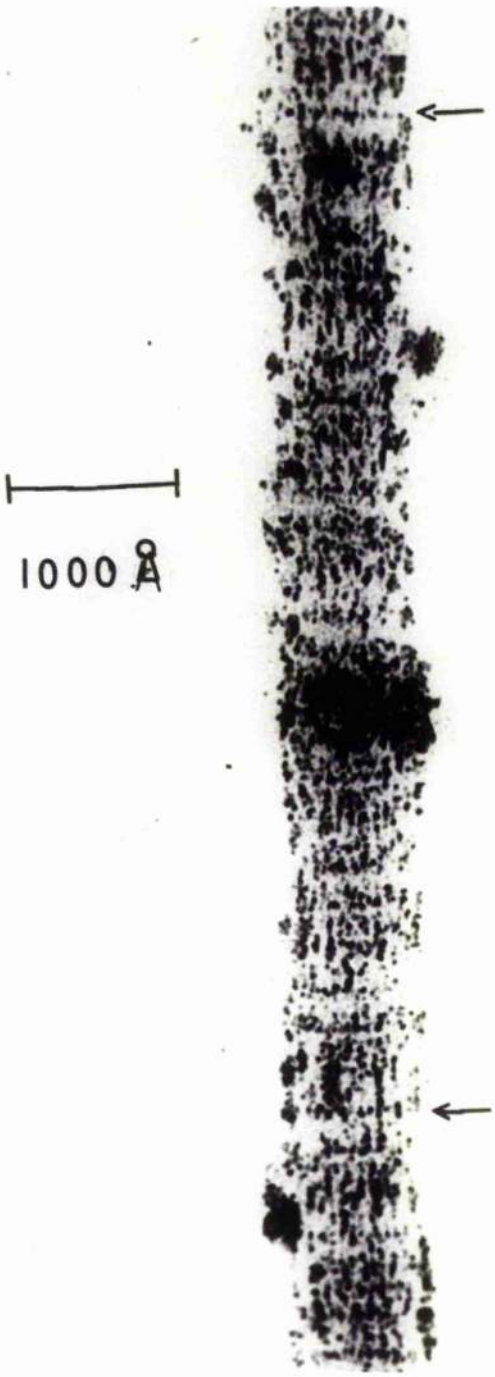
In a real sense, all electron micrographs are artifacts which require a sound conceptual background for their interpretation. In the present case it is considered that as a result of sulphate crosslinking, each polysaccharide chain adopts a coiled conformation which concentrates the bound bismuth sufficiently to produce an electron-dense spot. The size of the spot cannot be regarded as an absolute measure of the dimensions of the coiled chains because, if part of the chain is not sufficiently coiled to produce a threshold concentration of bismuth, that part

Figure 4



Monolayer of proteoglycan. Bismuth nitrate staining.

Figure 5



Complex formed at pH 5.5 between collagen and bovine nasal proteoglycan. Arrows indicate transverse orientation of some proteoglycan macromolecules. Bismuth nitrate staining.

Figure 6

Complex of collagen and heparin formed at pH 6.5.

Bismuth nitrate staining.

Figure 7

Complex of collagen and ChS formed at pH 6.5.
Bismuth nitrate staining.

will not be visible. Despite this qualification, it is a fact that there is very considerable uniformity in the size of the spots. Thus, in the macromolecules in Figure 4 the protein core is unstained and each 30 Å spot represents a "doublet" of chondroitin sulphate having a molecular weight of 15,000 - 18,000 (Meyer, 1966).

From a quantitative point of view, there are two drawbacks to this method. Firstly, the tendency of bismuth staining to detach bound polysaccharide chains from some areas of the collagen fibrils , and, secondly, the occurrence of some degree of entanglement of free macromolecules, as already discussed. Despite these two limitations, it is considered that electron microscopy after bismuth staining can reasonably be interpreted as giving a useful indication of the organisation of the complexes.

In general, at low pH values the glycosaminoglycans are distributed in a rather random fashion over the whole collagen period. At higher pH values, the binding pattern begins to exhibit a certain order, in particular the concentration of bound polysaccharides to a well defined site in each collagen period. Figure 5 illustrates the binding pattern of proteoglycan macromolecules at pH 5.5 (pH which produced maximum binding). The polysaccharide

chains are visualized as 30 Å spots, and the disposition of these spots strongly suggests that some macromolecules have adopted an orientation transverse to the collagen fibril axis (arrows in Figure 5) whereas others have a longitudinal orientation quite distinct from the in vivo arrangement found in bovine cartilage (Smith and Serafini-Fraccasini, 1968). Above pH 6.0 the outstanding feature is a limitation of polysaccharide binding to the whole of a 300 Å zone in each collagen period. In Figure 6 it is evident that the 300 Å - wide binding sites are occupied by rows of coiled heparin molecules. The regular distribution of chondroitin sulphate chains on the surface of a collagen fibril at pH 6.5 is shown in Figure 7. Individual spots are not evident at the magnification employed.

In all preparations the presence of large particulate aggregates strongly suggests that in these areas the basic pattern described above is obscured by entanglement. No clear connection, however, is apparent between degree of entanglement and molecular weight of the macromolecules involved.

The examination of a titration curve of collagen (Bowes and Kenten, 1948) shows that at low ionic strength, the net charge is practically zero above pH 4 so that one might be led to assume that no electrostatic binding could

take place between collagen and sulphated polysaccharides around physiological pH values. However, both cationic and anionic amino acid are localized in narrow zones within the collagen period, as indicated by electron microscopy and by amino acid sequential studies, and these charged zones are separated by non-polar regions. This arrangement introduces the possibility that alternating regions of positive and negative overall charge could exist even at pH near the isoelectric point. The consequent presence of an inhomogeneous electrical field would then create forces which cause an orientated movement of charged macromolecules towards the centres of inhomogeneity. Following such an interpretation it is suggested that the collagen used in the uptake experiments at pH above 6 contains an approximately 300 Å-wide zone of overall positivity. An "optimum" charge distribution at around pH 6 could then explain the peaking effect described for the uptake of both chondroitin sulphate and chondroitin sulphate - protein complex together with the orderly arrangement of macromolecules observed in the electron micrographs. In the case of these two larger macromolecules, a more regular packing on the surface of the collagen fibril would then in fact maximize the utilization of the fixed binding sites, even although these are restricted to a particular

zone in each collagen period.

PART TWO

Materials and Methods

Extraction of proteoglycan from bovine nasal cartilage.

Bovine nasal septa were obtained within 1 hour of slaughter, rinsed with water, cut into strips, and dehydrated with acetone. The strips were then ground under acetone to form a powder. The chondromucoprotein was extracted by the method of Malawista and Schubert(1958), care being taken to ensure that the temperature of the suspension did not rise above 4° C. The extract was fractionated by the method of Gerber, Franklin and Schubert (1960). Following homogenization in 0.15 M KCl, the solution was centrifuged for 1 hour at 78,000 g. The protoeglycan was then precipitated from the supernatant by ethanol. After washing with more ethanol it was redissolved in 0.15 M KCl and recentrifuged at 78,000 g for 1 hour. The material was reprecipitate with ethanol, the supernatant removed , and redissolved in 30% ethanol. Finally, the proteoglycan was precipitated by addition of a strong aqueous solution of $CaCl_2$ and washed three times with ethanol. The material was dried and stored in a dessicator. Before any experiments were carried out with the proteoglycan a standard procedure was adopted of solubilization over 24 hours at 4°C followed by centrifugation at 78,000 g for 1 hour to remove any aggregated material

Chemical treatment of the proteoglycan.

The PG was treated with freshly prepared dry 0.1 M HCl/ acetone for 1 hour at room temperature (20°C) as described by Serafini-Fracassini (1968). Early attempts at complete solubilization of the dried material proved unsuccessful. Subsequently it was found that the treated PG was completely soluble if it was dissolved immediately after collection on the teflon filter. It seems likely that this phenomenon is connected with the contraction that occurs during the drying process for, similarly, rapid dehydration of PG with acetone causes the material to contract into a dense mass that is soluble only with great difficulty. However, in this case the dried material was undissolved after stirring in an excess of 2% KCl at 4° C for 1 week.

Ultrafiltration of the chemically treated proteoglucan.

This procedure was adopted as a separation method because it is capable of being used as a continuous process, as against batch techniques such as gel chromatography. In the latter case, Agarose gel is particularly suitable for fractionation in the range 100,000 to 1,000,000 molecular weight, but high cost and technical difficulties preclude its use for larger scale preparations (ie. over 50 mg loading). Since physical charaterisation such as light scattering or osmometry is impossible on very small quantities of material, ultra-filtration seemed to provide a solution to this problem.

The filtration was carried out in 50 ml cells (Amicon Ltd.) using a reservoir allowing continuous filtration. The system being pressurised by nitrogen at 50 psi. The filters were obtained from either Millipore (Millipore Corp., Mass.) or from Sartorius

From information supplied by the Manufacturers, it was found that both maked of filters were identical in composition, being either cellulose acetate or cellulose nitrate. The chemically treated proteoglycan was diluted in 2% KCl to a concentration of about 0.5 mg ml^{-1} to facilitate filtration which was carried out using 2,000 Å ; 1,000 Å; 500 Å and 200 - 350 Å filters in that order. The filtrate obtained in each case was washed in situ with about 200 ml of solvent,

collected, and then dialyzed against water to remove excess salt and lyophilized prior to analysis.

The fractions obtained were as follows:

F1; not retained by a 200 - 350 Å filter.

F2; not retained by a 500 Å filter but retained by the 200 - filter.

F3; not retained by a 1000 Å filter but retained by a 500 Å f

F4; a trace amount only, not retained by a 2000 Å filter but retained by a 1000 Å filter.

F5; this was the total material retained by the 2000 Å filter

All filtration operations were carried out in a refrigerator at 4° C.

Light Scattering.

Light scattering measurements at 546 nm were performed at 20° C (room temperature) with a Brice Phoenix photometer. Absolute turbidities were determined by the working standard method (Brice et al., 1950). The instrumental proportionality constant was determined from the equation given by Brice et al. - with the necessary data provided by the manufacturers. The working standard constant a which relates the working standard to an opal diffusor was always redetermined prior to any set of experiments.

All dilutions on material were carried out in a dust box which was constructed from perspex with inserted plastic gloves to allow manipulations within the box. By trial and error it was found that dust was most effectively removed by liberally smearing the walls of the container with vaseline; a closed circuit air pump then circulated the air through the box and additional filters and grease traps for about ten minutes prior to any operation. Dust was removed from solutions by both filtration through Millipore filters (Millipore Corp.,Mass., U.S.A.) and high speed centrifugation. For solvents, repeated filtration through 220 nm pore size filters (three times) gave satisfactory results. Glassware and cells were washed in an 'acetone washer' for a minimum period of three hours.

Since for very low scattering ratios dissymmetry values Z are misleading due to emphasised reflection effects, Z values cannot be used as a criterion for dust free solvents. Instead, values for the absolute turbidity of less than $3.0 \cdot 10^{-5}$ cm^{-1} were taken as being indicative of adequate solvent clarification. In most cases, scattering measurements were first made on solvent, the material then being filtered directly into the cell after prior clarification. Samples were withdrawn for analysis immediately after scattering measurements.

Measurement of specific refractive index increment ($\Delta n/\Delta c$).

These values were determined at 546 nm with a differential refractometer (Polymer Consultants Ltd.). The instrument was calibrated with solutions of 2% sucrose using a Δn value of 0.002860 (Browne and Zerban, 1941). The constant K was found to be $9.659 \cdot 10^{-7}$ ml g^{-1} and the following equation was used :

$$\Delta n = K \Delta d$$

where the total deviation Δd is given by

$$\Delta d = (d^{0}soln - d^{180}soln) - (d^{0}solv - d^{180}solv)$$

For each value of d, ten readings were averaged, the values accepted being within the range $\pm 5 \cdot 10^{-6}$, this resulting in a limiting accuracy of $1 \cdot 10^{-5}$. However, generally it was possible to obtain readings with a reproducibility within the range $\pm 5 \cdot 10^{-6}$ or slightly better, resulting in an error of approximately 1% in the measured $\Delta n/\Delta c$.

Determination of the molecular weight of serum albumin.

This was carried out as a check on experimental technique. The concentrations of the albumin (Calbiochem A grade, bovine plasma, lot 901689, electrophoretically pure) were determined spectrophotometrically from measurements of the optical density at 276 nm. A standard curve was prepared based on dry weights. Using an experimentally determined moisture content of $9.0 \cdot \pm 0.3\%$, it was found that reliable results were obtained within the range 0.02 - 0.15%. $\Delta n/\Delta c$ was determined for a number of concentrations and was found to be constant within the range 0.3 - 3.0% and to have a value $0.185 \pm 0.6\%$ taking into account the moisture content of 9%. This value is in agreement with those reported by Halwer et al., (1951).

Light scattering was carried out on solutions of albumin in 0.1 M NaCl by measuring the scattering at 90° in a semi-octagonal cell. The molecular weight was obtained by plotting

Hc/τ against concentration (Figure 8). The intercept (by least squares) was found to be 1.46, corresponding to a molecular weight of 68,500 - a figure well within the published estimates for this protein. (Tietze and Neurath, 1951, 68,000 - 71,000 ; Scatchard et al., 1946, 69,000 by osmometry).

Characterisation of extracted proteoglycan.

Angular scattering intensities of unpolarized light were measured over the range 35° to 135° using a cylindrical cell. The cell was calibrated by determining the ratio of 90° scatter of 0.03% PG in the cylindrical cell to that in the previously calibrated semi-octagonal cell. The possibility of distortion of the scattering envelope was investigated by measuring the scattering of a solution of fluorescein (1 γ per ml). Since the fluorescence radiation of fluorescein consists entirely of the vertical component of polarized light, the angular intensity should be symmetrical around the cell. The angular distribution (in arbitrary units) multiplied by $\sin\theta$ to correct for the volume viewed is shown in Figure 9. It can be seen that the observed intensity was independent of angle from 40° to 135°. A solution of the proteoglycan in 0.268 M KCl (2%) was clarified by centrifugation at 40,000 g for three hours and filtration

through a 450 nm filter. The Rayleigh ratio was determined by means of the equation given by Tomimatsu and Palmer (1963). The equation incorporates corrections for the scattering of the reflected portion of the primary beam, the attenuation of scattered light at an angle θ by reflection at the air/glass interface at the point of measurement, and the contribution of reflection in the θ direction and of the light scattered in the $+(180 - \theta)$ direction. The data was treated according to the method of Zimm(1948). For PG, an average value of $\Delta n/\Delta c$ was found to be 0.158 ml g^{-1} which leads to a value of K equal to $\frac{2\pi^2 n_o^2 (\Delta n/\Delta c)^2}{N\lambda}$ of $1.64 \cdot 10^{-7}$. Concentrations were determined both by reference to a standard graph of absorption at 275 nm and, at very low concentrations, by weighed dilutions. That is, the cell was weighed before and after addition of solvent. Stirring was carried out by means of a magnetic flea - the photometer being equipped with a stirring device.

The absorption plot was prepared on the basis of a dry weight estimation averaged from four estimations on a solution of proteoglycan dialyzed exhaustively against water.

Figure 8

Plot of Hc/τ against c for serum albumin.
H is a constant
c is the concentration in gram per cent
τ is the turbidity
Further details are given pages 35-36.

FIGURE 8

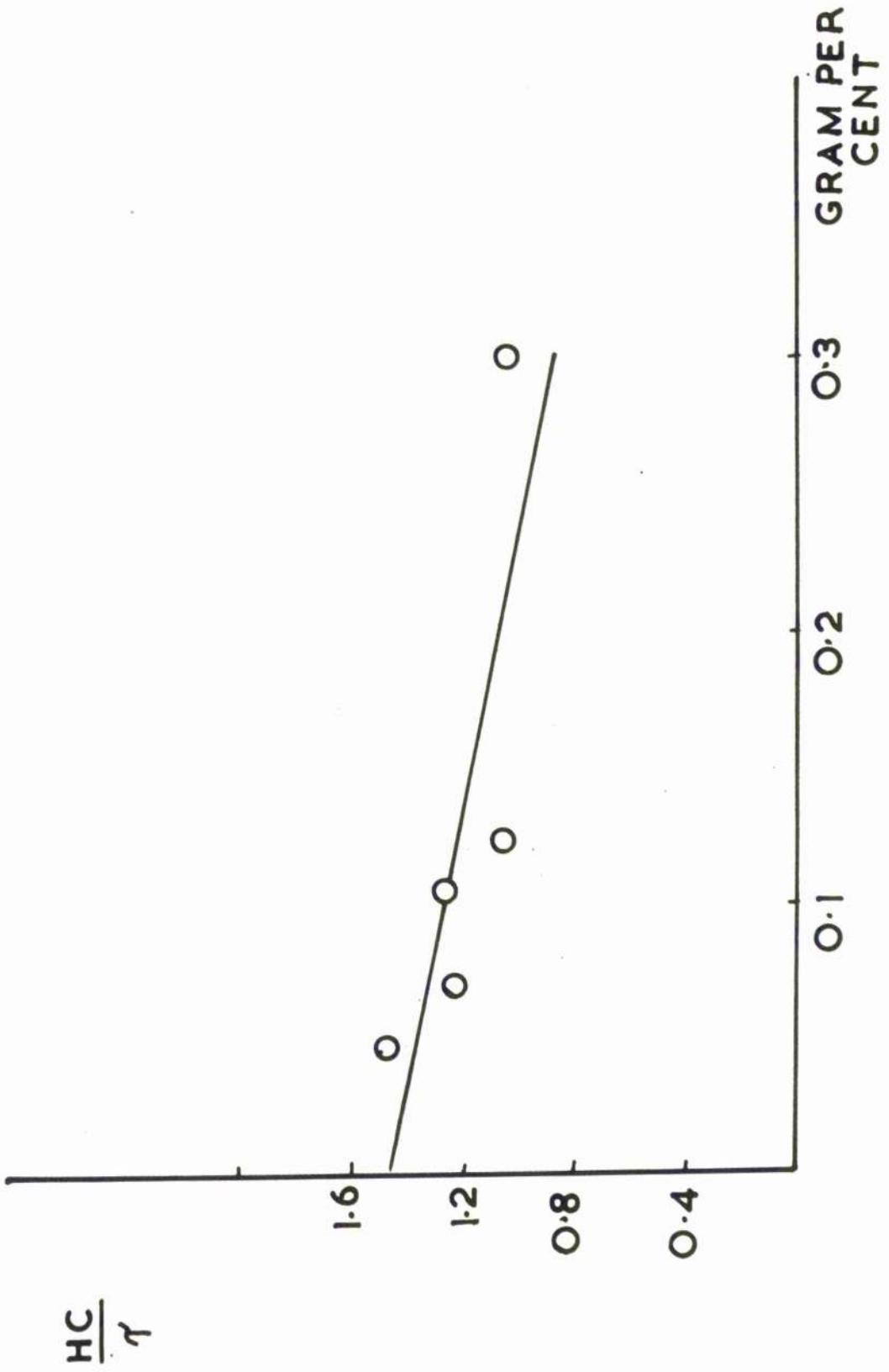


Figure 9

I . sin Θ as a function of angle Θ for a solution of fluorescein in water. Details and explanation of symbols is given on page 36.

FIGURE 9

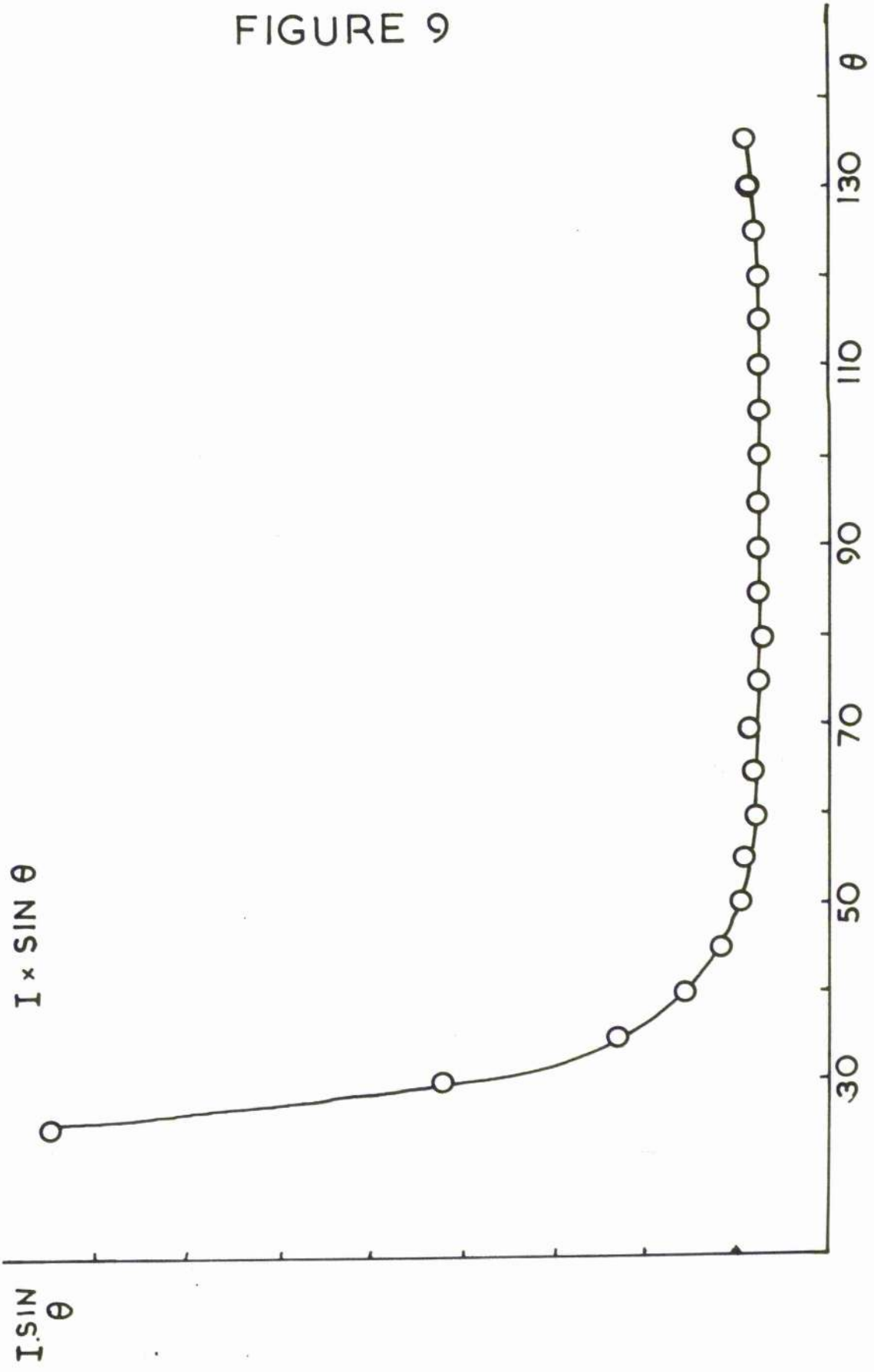


Figure 10

Zimm plot prepared from data obtained from measurements of scattering intensity on solutions of extracted and purified PG.

The four concentrations used were:-

1. 0.01479 %
2. 0.02101 %
3. 0.02600 %
4. 0.03182 %

K is a constant
c is the concentration
R_θ *is the Rayleigh ratio at angle* θ

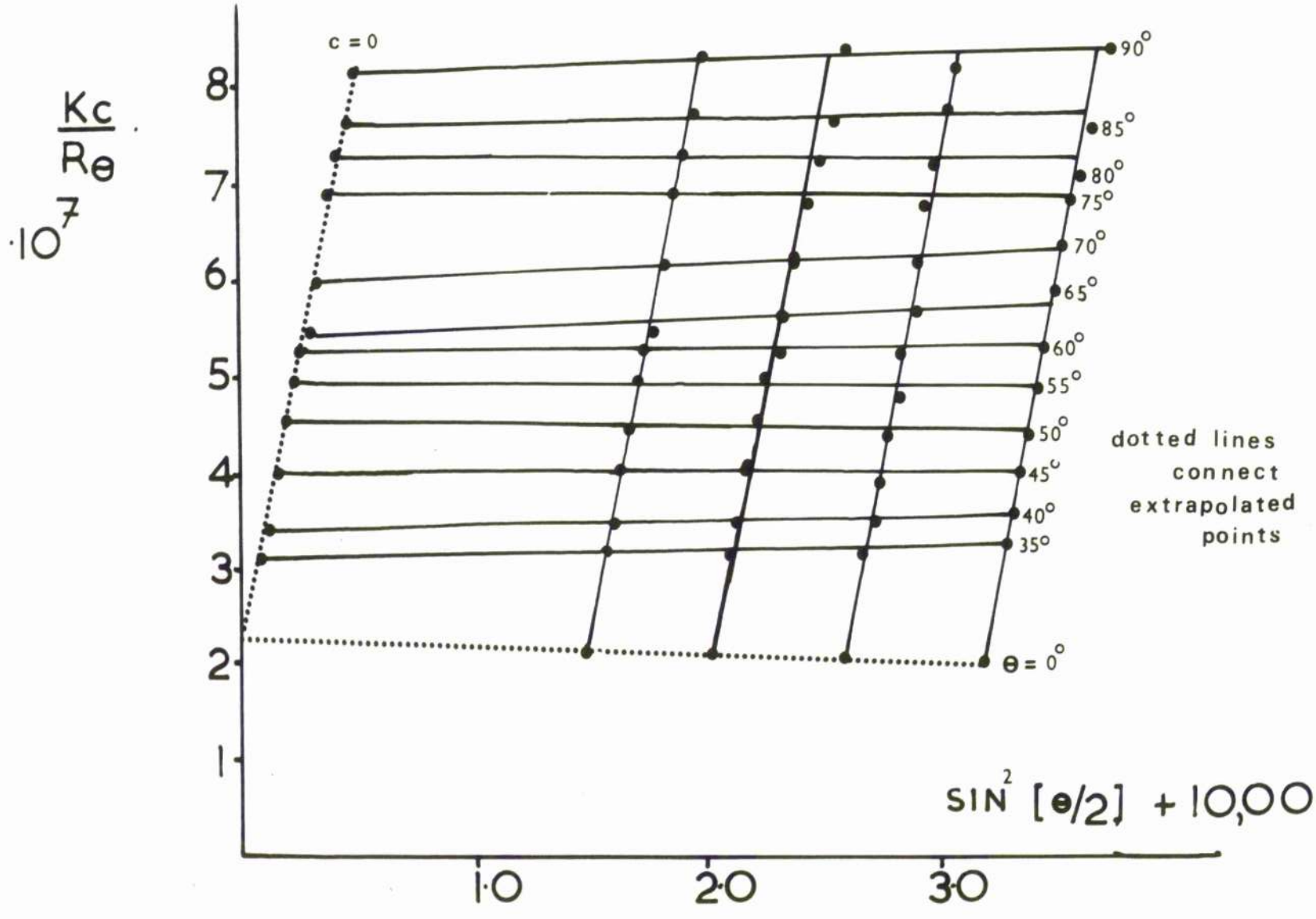

Kc/Rθ
·10⁷
8
7
6
5
4
3
2
1
c = 0
90°
85°
80°
75°
70°
65°
60°
55°
50°
45°
40°
35°
θ = 0°
dotted lines connect extrapolated points
1·0
2·0
3·0
SIN² [θ/2] + 10,000.c

FIGURE 10

Ultracentrifugal analysis.

Ultracentrifugation was performed in a Spinco model E ultracentrifuge. All experiments were carried out at 20.0.°C in either an AnH or AnJ rotor.

Sedimentation velocity.

Sedimentation coefficients were determined in a 12 mm single sector cell or in a 12 mm capillary type synthetic boundary cell. Schlieren patterns were recorded on photographic plates and analysed on a microcomparator (adjustment possible down to 1μm). Apparent sedimentation coefficients were corrected to standard conditions.

Sedimentation equilibrium.

Sedimentation equilibrium studies were carried out with a 12 mm double sector cell with sapphire windows using the Rayleigh interference optical system. The meniscus depletion equilibrium method of Yphantis (1964) was used in preference to the more comventional low speed techniques. To aid discussion of the advantages and limitations of this method some relevant theory is included at this point.

In the presence of an externally applied ultracentrifugal field, the criterion for equilibrium of a system is

that the total potential of any constituent is constant at each radial position in the cell. Using this criterion and ignoring pressure dependence, the basic sedimentation equilibrium equation may be derived for a binary system (Van Holde and Baldwin, 1958)

$$\frac{M(1 - \bar{v}\rho)\omega^2 r \cdot c}{RT} = \frac{dc}{dr} \cdot (1 + c(d\ln\gamma/dc))$$

where the final term on the right hand side allows for nonideality.

r is the radial position
c is the concentration at that radial position
v is the partial specific volume of the solute
ρ is the density of the solution
γ is the activity coefficient of the solute on the concentration scale

Temporarily neglecting the non-ideality term, this equation in terms of the concentration gradient can be integrated from the meniscus $\underline{a}$ to the point $\underline{r}$ to give an equation for the concentration at any point.

$$\int_a^r 1/c(r)\, dc(r) = \frac{M(1 - \bar{v}\rho)\omega^2}{RT} \int_a^r r dr$$

and $$\ln \frac{c(r)}{c(a)} = \frac{M(1 - \bar{v}\rho)\omega^2}{RT} \cdot \frac{r^2 - a^2}{2}$$

Taking antilogs:

$$c(r) = c(a) \cdot \frac{\exp M(1 - \bar{v}\rho)}{RT} \; \frac{a^2 - r^2}{2}$$

where M is in this case the weight average molecular weight. For low speed studies in a sector shaped cell, assumption of conservation of mass allows manipulation of this equation to give an expression utilizing the difference in concentration between the meniscus a and the base of the cell b together with C_o where this is the initial concentration (Williams et al., 1958).

$$\frac{c(b) - c(a)}{C_o} = \frac{M(1 - \bar{v}\rho)\omega^2}{RT} \cdot b^2 - a^2$$

The quantity $c(b) - c(a)$ will be proportional to the total number of fringes crossed in progressing from the meniscus to the base of the column. To obtain the number of fringes proportional to C_o a synthetic boundary experiment must be performed in which solvent is layered over the solution whereupon the fringe shift can be measured accurately.

(Lansing and Kraemer, 1936).

An alternative fringe calibration method has been suggested by Richards and Schachman (1959). In this method the point in the cell is found at which the concentration of solute does not change with time - that is, the concentration is equal to C_o. This so-called "hinge point" may be found relative to the zero order fringe by a procedure detailed in the paper. Difficulties in unambiguously locating the zero order fringe and the necessity of closely matching the refractive indices of reference and sample solutions are considerable drawbacks to this technique.

As mentioned above, the integrated equation for low speed studies assumes conservation of solute; however Yphantis (1964) has pointed out that in dilute solutions this may not be justified. If this is so then the apparent weight average molecular weight determined will be too low. Solute may be removed in two ways.

1. Aggregation and subsequent deposition.
2. Adsorption on to the cell walls.

Aggregation will result in base thickening which is easily checked. Adsorption however may be undetectable. Experiments by Yphantis (1960) have shown that significant amounts of solute may be adsorbed at concentrations around 0.05% and below, that is, about 0.5 mg per ml since the double sector

cells used in equilibrium studies have a comparatively high surface area to volume ratio. These experiments therefore show that in dilute solutions conservation of solute cannot be guaranteed and as a result there is a need for a precise concentration reference at some point in the cell. This may easily be established if the speed is sufficiently high to cause depletion of solute from the meniscus. If the concentration of solute at the meniscus is zero then concentrations in the cell will be proportional to the refractive index difference between the region near the meniscus and the point of interest. Apart from overcoming this concentration reference problem a number of other advantages are gained by this procedure :

1. The resultant increase in concentration towards the base of the column means that measurements on initially more dilute solutions are possible.
2. Since the degree of concentration is a function of the effective molecular weight of a species making up a solution, a partial size fractionation can result which in certain cases can result in estimates of the smallest component comprising a solution.
3. It is possible to obtain estimates of the number average molecular weight, for Lansing and Kraemer (1935) pointed out that $\bar{M}n$ can only be calculated

directly from sedimentation equilibrium data if the solute concentration approaches zero at some point in the cell.

4. Concentration dependent systems may be studied in a single run, for the gradient will range from zero at the meniscus to about 0.1% for a sample loading of 0.05%.
5. Time required for attainment of equilibrium is reduced - this being a very considerable advantage in a busy department.

One disadvantage of this method lies in the limiting accuracy of about 1 - 2 %. This, however, is of minor importance compared to the power of the technique in analysis of non-ideal poly disperse systems as, for example, in the present case.

Adopted procedure.

It was found as a rule that equilibrium was attained in about twenty hours, but its presence was always checked by comparison of plates taken at hourly intervals. The criterion for depletion was taken as the absence of fringe displacement for at least 1/3 of the column height as in Figure 11 which shows the plate for fraction 1.

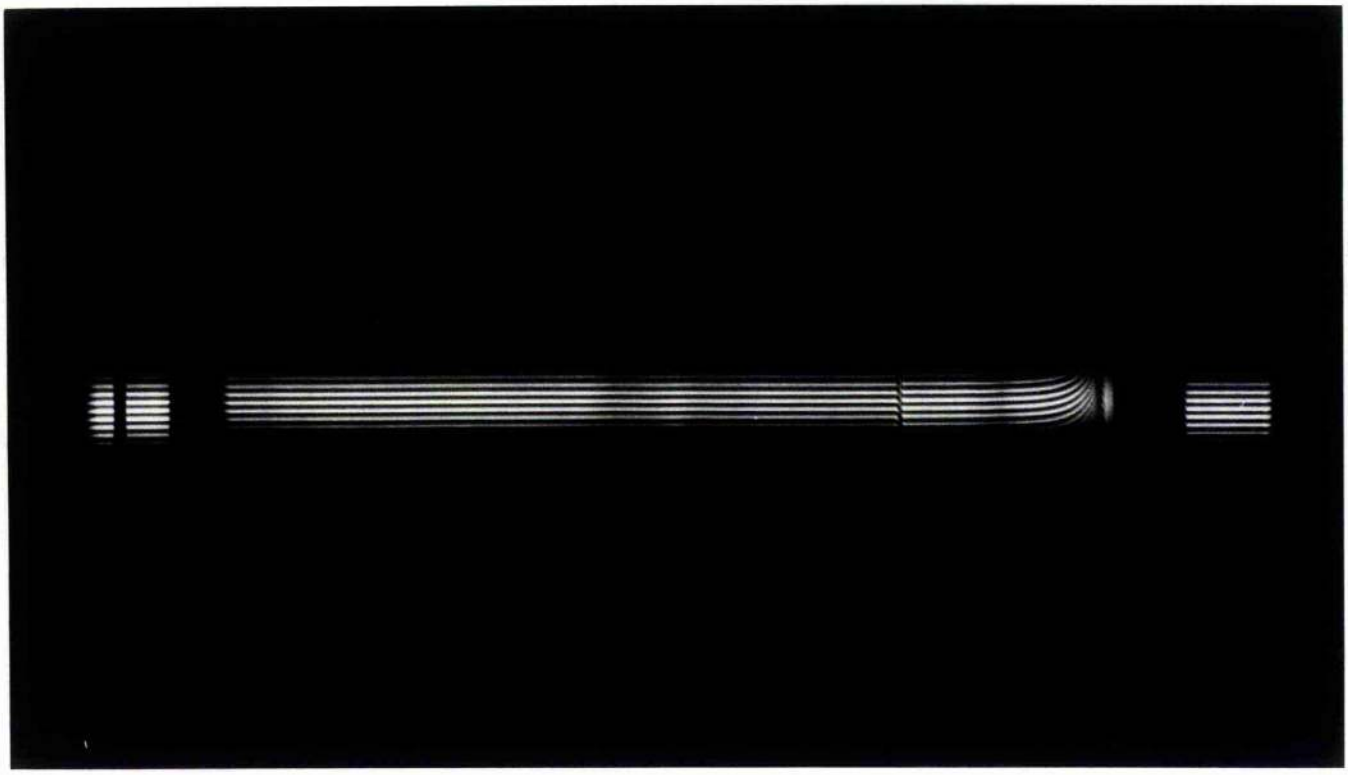
Results were plotted in the form of log (Yr - Yo) (Yr - Yo is the net fringe displacement), against R^2 (the distance of that point from the centre of rotation). Values for the point average effective reduced weight average molecular weights defined as $\alpha_\omega(r)$ where this is :-

$$\alpha_\omega(r) = \frac{M(1 - \bar{v}p)\omega^2}{2RT \cdot 2.303},$$

were then plotted against R^2. These were derived by determination via least squares of the slope of the straight line through successive groups of five poinis. $\bar{M}_z$ over the whole cell was obtained by extrapolation of $\alpha_\omega(r)$ values to the base of the column. Similarly, where possible, $(\bar{M}w)c \to o$ values were obtained by extrapolation to the meniscus. In all cases the extrapolations were performed by least squares regression. In the log (Yr - Yo) / R^2 plots, since polydispersity will result in upward curvature and non-ideality in downward curvature, it is to be expecied that material exhibiting both these characteristics (such as fractions of glycosaminoglycans), might well yield a resultant straight line. For this reason, material was examined, where possible, at high (about 0.6 mg ml^{-1}) and low concentration (0.2 mg ml^{-1}

To reduce unforeseem complications arising from interactions between components 2, 3 and 4 etc., the runs were

Figure 11



Appearance of the interference fringes at equilibrium in a meniscus depletion experiment. The example shown is that for fraction 1 ; the results are plotted in Figures 19 and 20.

carried out in nonbuffered solutions at the isoelectric point of the sample. The pH of a solution of proteoglycan (0.5 mg ml^{-1}) was about 4 and was found not to vary significantly on standing.

Determination of partial specific volume.

Apparent partial specific volumes were calculated from density measurements carried out in a density gradient column (Linderstrom-Lang and Lanz, 1935). The column was prepared from mixtures of bromobenzene (freshly distilled) and kerosene, to give a gradient over the range 0.99 - 1.03 sg - care being taken to presaturate the column with water.

A precision syringe was used to introduce drops into the column (average volume about 0.5µl) and the equilibrium positon of the drops was determined using a travelling microscope with reproducibility in the order ± 50 µm, giving a limiting accuracy of 1%. It was found that slight variation in droplet size made no detectable difference to the equilibri position. The column enclosed in a water jacket was maintaine at a temperature of 20.00 ± 0.01°C. The apparent partial specific volume was calculated from the relation :

$$\bar{v} = \frac{100/d - (100 - n)/d_o}{n}$$

where d is given by

$$d = d_1 + \frac{(h - h_1)}{(h_2 - h_1)} \cdot (d_2 - d_1)$$

d is the density of the solution.

d_o is the density of the solvent.

d_1 is the density of a sucrose standard and h_1 its posi

d_2 is the density of the next standard.

n is the concentration in g/100 ml.

The column was calibrated by using solutions of sucrose of known density. Approximately 5% sucrose was made on a litre scale to minimise errors and diluted in 100 ml portions. The densities of these solutions were estimated by interpolation on a graph prepared from data for the densities of 2 - 5% sucrose from the International Critical Tables.

The apparent values of $\bar{v}$ calculated for glycosaminoglycans are assumed, for this work, to be concentration independent - that is, these solutions are assumed to be ideal. This is probably an incorrect assumption, however, it is considered that the accuracy obtainable using the density gradient column does not justify measurements allowing exr polation of $\bar{v}$ to zero concentration.

Since the glycosaminoglycans and related macromolecules

are polyelectrolytes with very high charge to weight ratios, the presence in solution of a reasonably high concentration of third component (greater than about 0.2 I) is necessary to reduce the effects of non-ideality. As a result of this, interactions between this third component and the macromolecul will alter its apparent molecular weight. However, Casassa and Eisenberg, (1961), have shown that it is possible to derive the correct molecular weight without specifically evaluating this interaction. They have derived the following expression for $\bar{v}^*$, the true partial specific volume.

$$\bar{v}^* = \frac{1}{1 + T}\left(\bar{v} + \frac{T}{d_o}\right)$$

where $\bar{v}$ is the apparent partial specific volume calculated from density measurements on a solution of component 2 dialyzed exhaustively against a solution of the third component. The problem thus amounts to finding a value for T which is the fraction of associated component 3. For the values of $\bar{v}^*$ used here, T has been estimated from dry weight estimations on equilibrium dialyzed material with checks by refractometry. It is clear that neither of these methods can give values comparable with the sensitivity of the density determinations, but, as yet, a solution to this problem has not been found. For the determinations on glycos-

aminoglycans, $\bar{v}$ values have been in the order 0.53 with values of $\bar{v}^*$ in the order 0.65. This is a considerable difference in view of the effect this will have on the apparent molecular weight. Because of the reciprocal relation between M (the molecular weight) and $\bar{v}^*$, the importance of accurate determinations of the latter increases with molecular weight. As an illustration of the magnitude of this dependence, some values of $\bar{v}$ have been plotted against the corresponding apparent molecular weights obtained by using an arbitrary value of 0.65 cm^{-1} for the effective reduced $\bar{M}w$. These are shown in Figure 12. This perhaps rather obvious relationship is stressed in view of the widespread practice of either assuming a value for $\bar{v}$ or apparently neglecting a possible correction for it.

Figure 12

Plot of $\bar{v}$ against apparent molecular weight using an α_ω value of 0.65 cm^{-1}. See page 52. Symbols are as defined in the text.

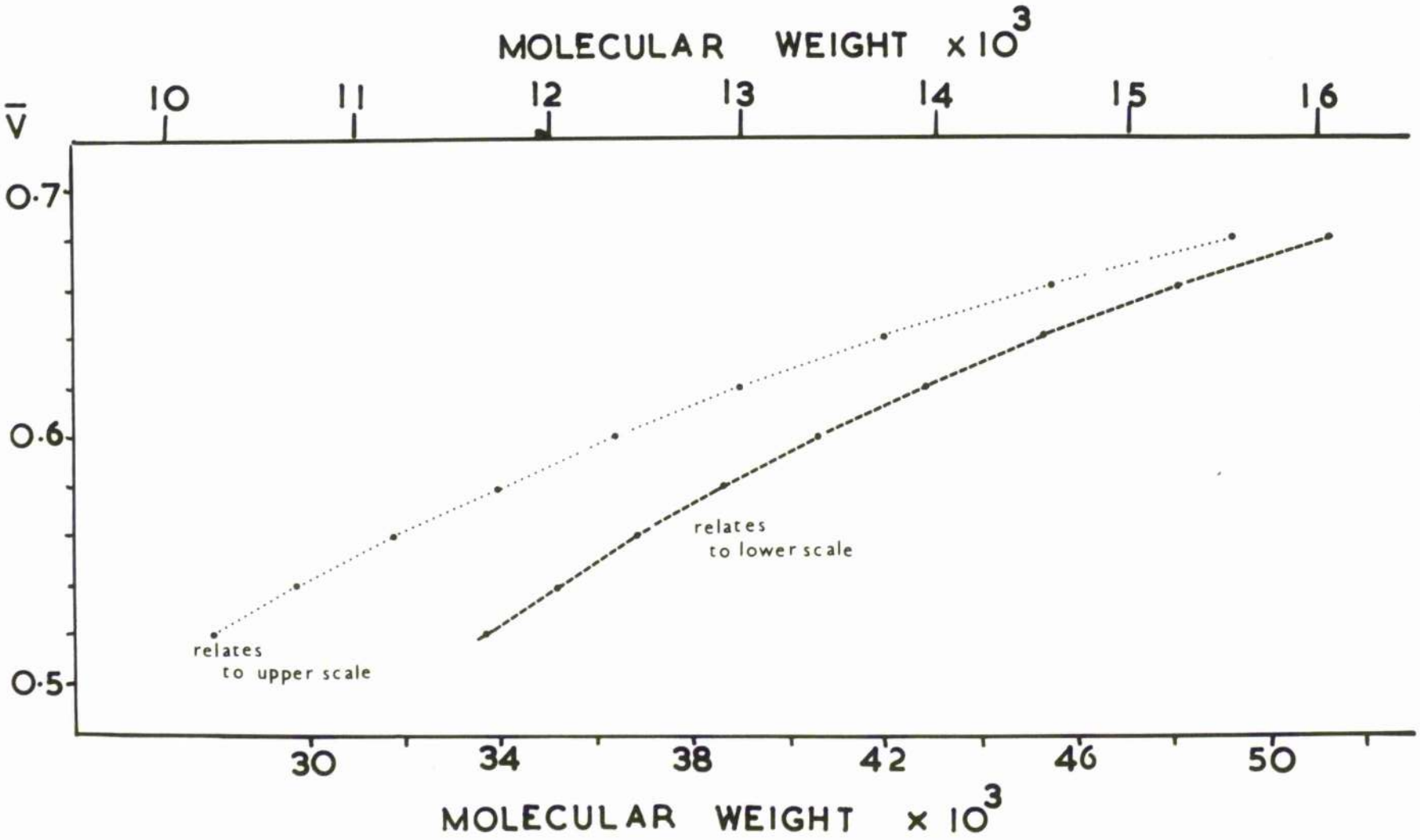

MOLECULAR WEIGHT x 10^3
10
11
12
13
14
15
16
V̄
0·7
0·6
0·5
relates to upper scale
relates to lower scale
30
34
38
42
46
50
MOLECULAR WEIGHT x 10^3

FIGURE 12

Osmometry.

A 503 high-speed membrane osmometer (Hewleit-Packard, Pa., U.S.A.) equipped with B-19 membranes (Schleicher and Schuell, Keene, Germany) was used to measure osmotic pressures. The constant temperature control was set at 20°C. To reduce the effects of Donnan equilibrium, determinations were carried out on samples dissolved in, and dialized against, 1 M KCl. A stock solution of material was prepared on the basis of dry weight and progressive dilutions were made from this. For each concentration four separate determinations were carried out. By careful weighings of the dialysis sac (with allowance for the wet weight of the tubing), the changes in volume of solution during dialysis were taken into account in estimating concentrations.

Amino acid analysis.

Solutions of the various samples in about 200 times their own weight of constant boiling HCl, were heated in sealed tube under vacuum at 110° for 24 hours. The samples were taken down to dryness in a rotary film evaporator, the temperature of the bath being maintained at 30° C. Amino acid analysis wa carried out with either a Technic on Auto-analyser or a Locart machine. Tryptophan was not analysed in view of its doubtful occurrence in PG. (Serafini-Fracassini et al., 1967). Hexosamines were estimated at the same time. Destruction due to hydrolysis of amino acids was corrected for by means of the coefficients derived by Serafini-Fracassini et al. (1967).

RESULTS.

Light scattering.

From Figure 10 the slope of the extrapolated zero concentration line is $11.98 \cdot 10^7$ and the common intercept is $2.25 \cdot 10^7$. The molecular weight is $4.4 \cdot 10^6$. Since for the two extrapolated lines the following relations apply:

$$\left(\frac{Kc}{R\theta}\right)_{\substack{c \to 0 \\ \theta = 0}} = \frac{1}{Mw} + 2Bc$$

$$\left(\frac{Kc}{R\theta}\right)_{\substack{c = 0 \\ \theta \to 0}} = \frac{1}{Mw}\left(1 + \frac{16\pi^2}{3\lambda^2} \cdot R_G^2 \sin^2\left(\frac{\theta}{2}\right)\right.$$

then the slope at zero concentration will be

$$\frac{16\pi^2}{3\lambda^2} \cdot R_G^2 \cdot \frac{1}{\text{intercept}} \cdot \sin^2\left(\frac{\theta}{2}\right)$$

$$\text{or } \sqrt{\frac{\text{initial slope}}{\text{intercept}}} = \sqrt{\frac{16}{3}} \cdot \pi \cdot \frac{R_G}{\lambda}$$

and the slope at zero θ is $2B/K$

From the graph, it is clear that the second virial coefficien B is negligible, showing that there is little interaction between molecules at the concentrations used.

R_G, the Z average radius of gyration, is 2,320 Å. This is on the assumption that the refractive index of the solution of PG is not significantly different from that of water - taken as 1.334. The value for λ was then taken as

5460 Å multiplied by 1.334 = 7283 . 6 Å

Ultracentrifugation.

Plots of log (Yr - Yo) / R^2 and $\alpha_\omega(r)$ / R^2 are given in Figures 13 to 28.

A preliminary experiment was carried out with chymotrypsin as an example of a fairly homogeneous and ideal sample. At the other extreme, alkali-extracted chondroitin sulphate was used as an example of a polydisperse and non-ideal materia Data on the various fractions are given in the legends facing the graphs. The lines indicating the column bases in some graphs are not accurately placed, but are put as guides. The menisci are omitted since they would be out of scale.

Osmometry

Results are given in Figure 29. The intercept is 1.436 which corresponds to a molecular weight of 20,120 ± 600 Due to lack of material, experiments were performed for fraction 1 only.

Figures 13 and 14

Sample: Chymotrypsin (Sigma) unfractionated.

Concentration = 0.54 mg ml^{-1} ; *phosphate buffer pH 7.2*

speed = 33,450

$\bar{v}$ = 0.721 *(Value taken from Schachman, 1963)*

$\bar{M}w$ = 25,480

$\bar{M}z$ = 26,900 ($\bar{M}z$ *over entire cell)*

The literature value is given as 24,500 (Schachman, 1963)

The extrapolation shown in Figure 14 is not intended to represent an extrapolation to the meniscus.

Although lines have been drawn on one or two graphs, they have generally been left out since it was felt that they tend to 'pre-judge the issue'.

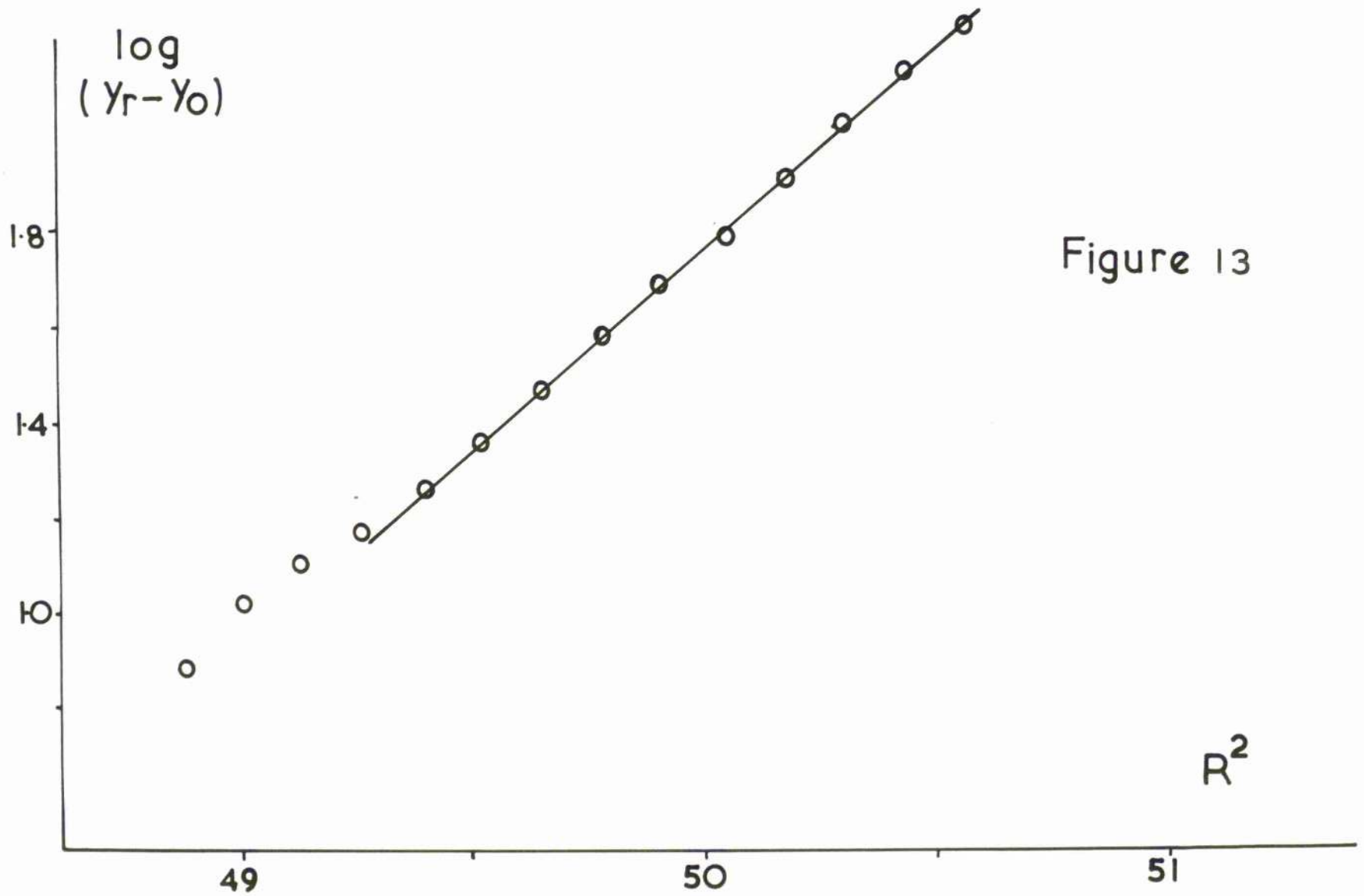
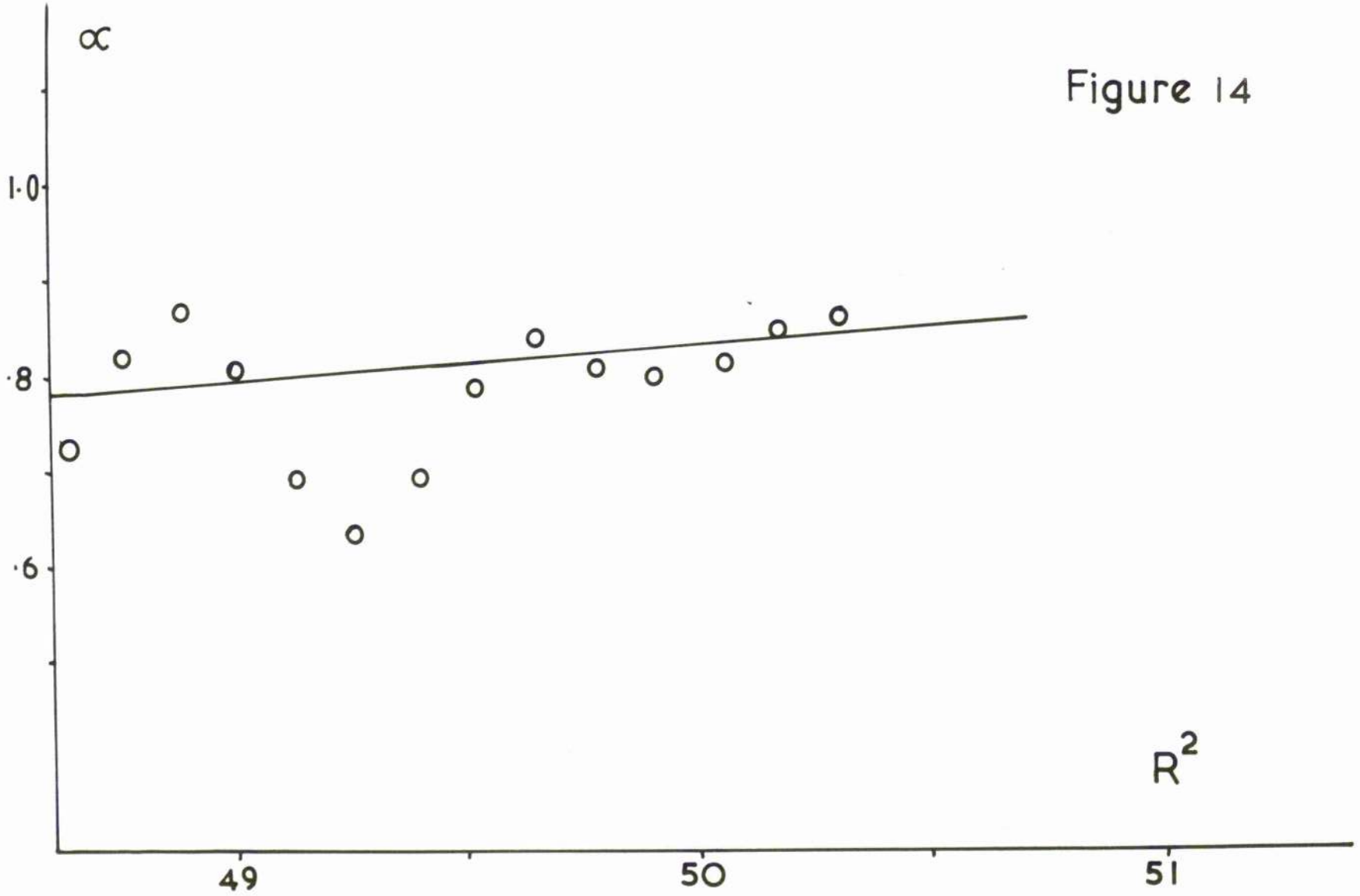


Figure 13

Figure 14



Figures 15 and 16

Sample: Chondroitin sulphate

concentration = 0.69 mg ml^{-1} *in 2% KCl*

speed = 21,740

$\bar{v}^*$ = 0.630

α_ω(base) = 0.915

$\overline{M}z$(base) = 58,300

α_ω(meniscus) = 0.497

$\overline{M}\omega$(") = 29,640

It will be noted that in the α_ω plots there is a 'dead' space at the base of the column. This results from the averaging procedure of using 5 points. In any case, estimations at the base of the column are suspect because of the difficulty in reading the fringe displacements. This only applies to readings within about 50μm from the oil. Beyond this region, the readings are very accurate (± 2μm) since they represent a relatively large % of the total displacement. The uncertainty rises again nearer the meniscus.

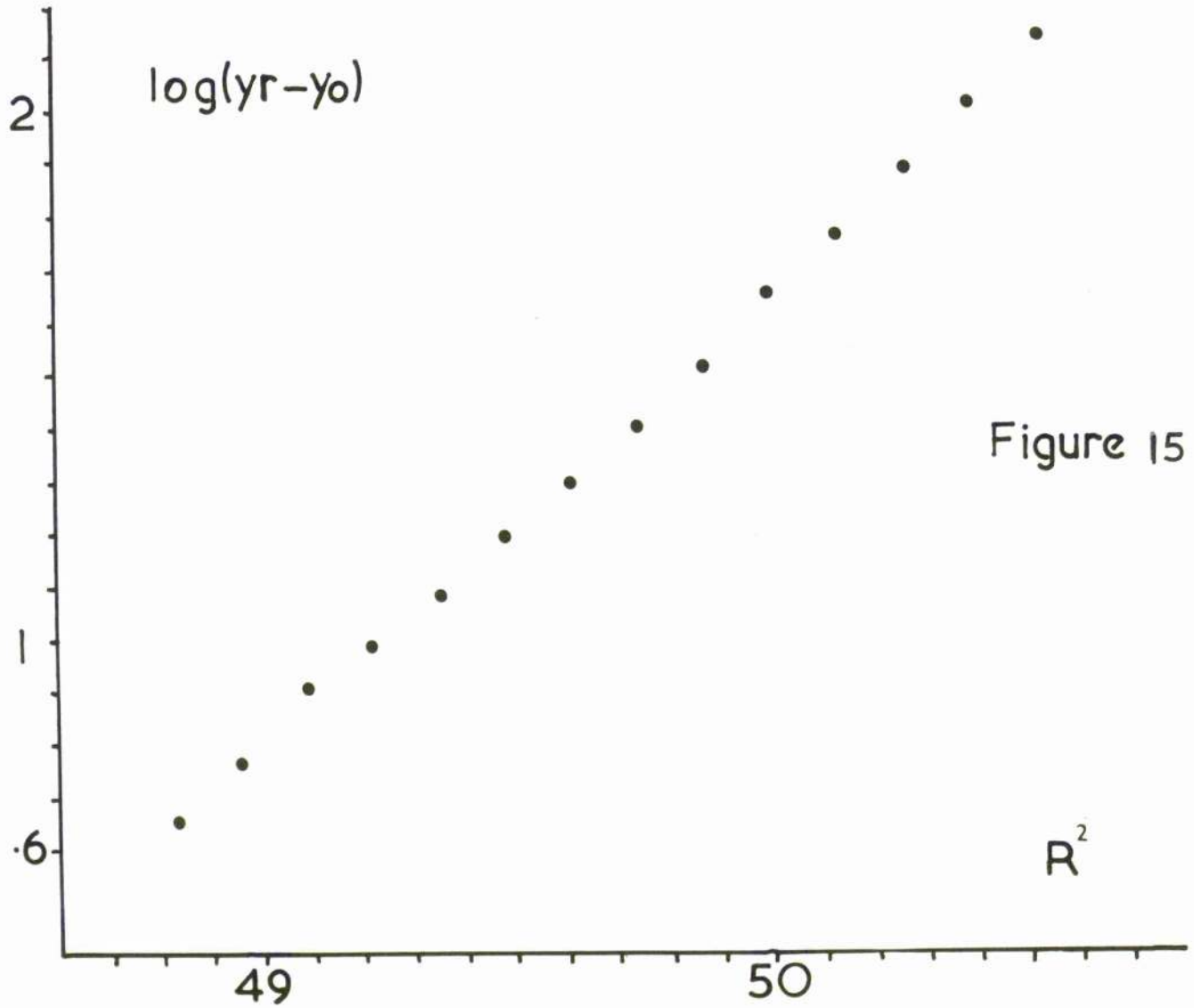


Figure 15

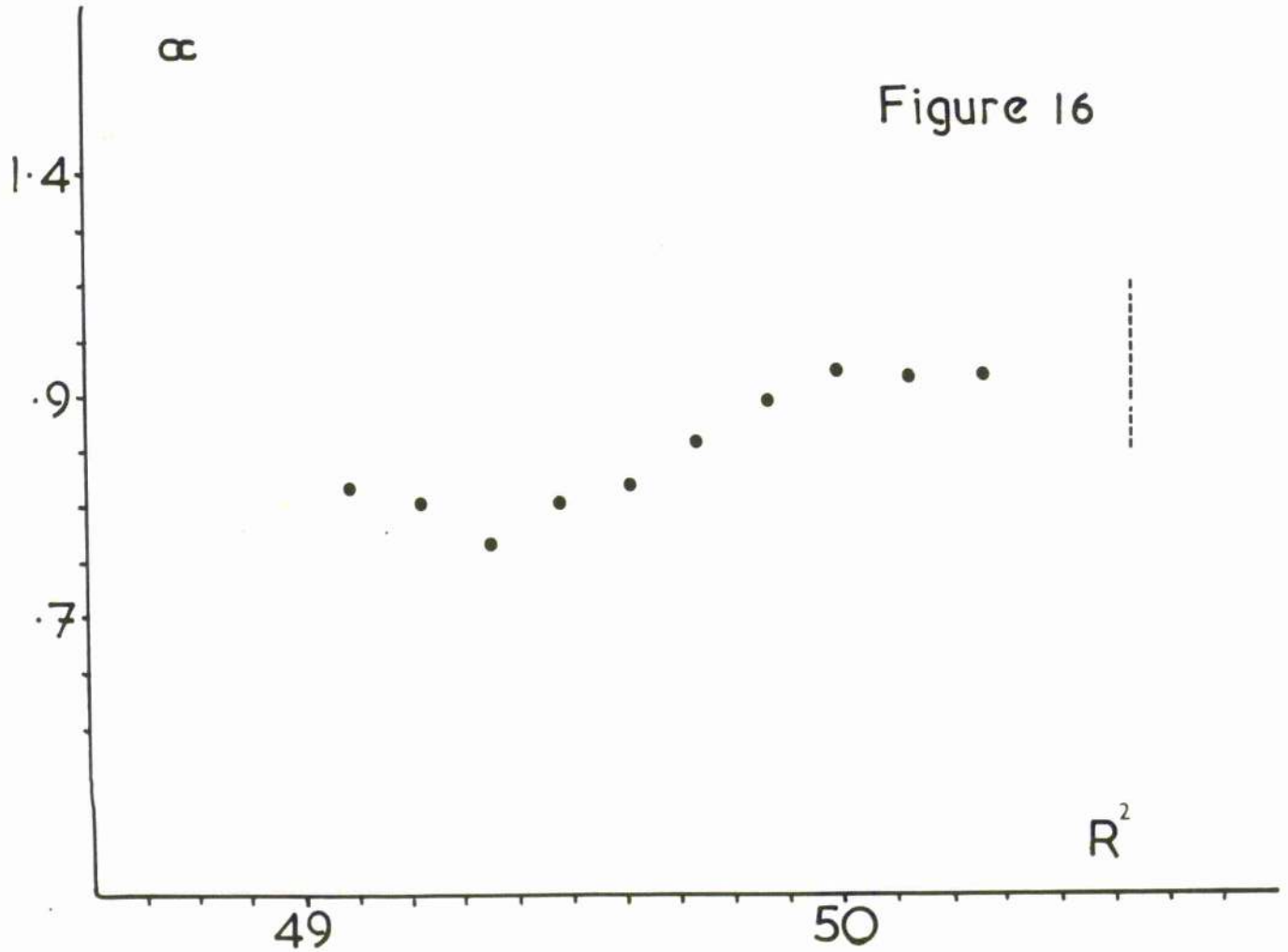


Figure 16

Figures 17 and 18

Sample: *Chondroitin sulphate*

concentration = 0.24 mg ml^{-1} in 2% KCl

speed = 21,740

$\bar{v}^*$ = 0.630

α_ω(base) = 1.084

$\bar{M}z$ = 64,650

α_ω average on linear points = 0.551

$\bar{M}w$ = 32,860

A meniscus extrapolation is not possible

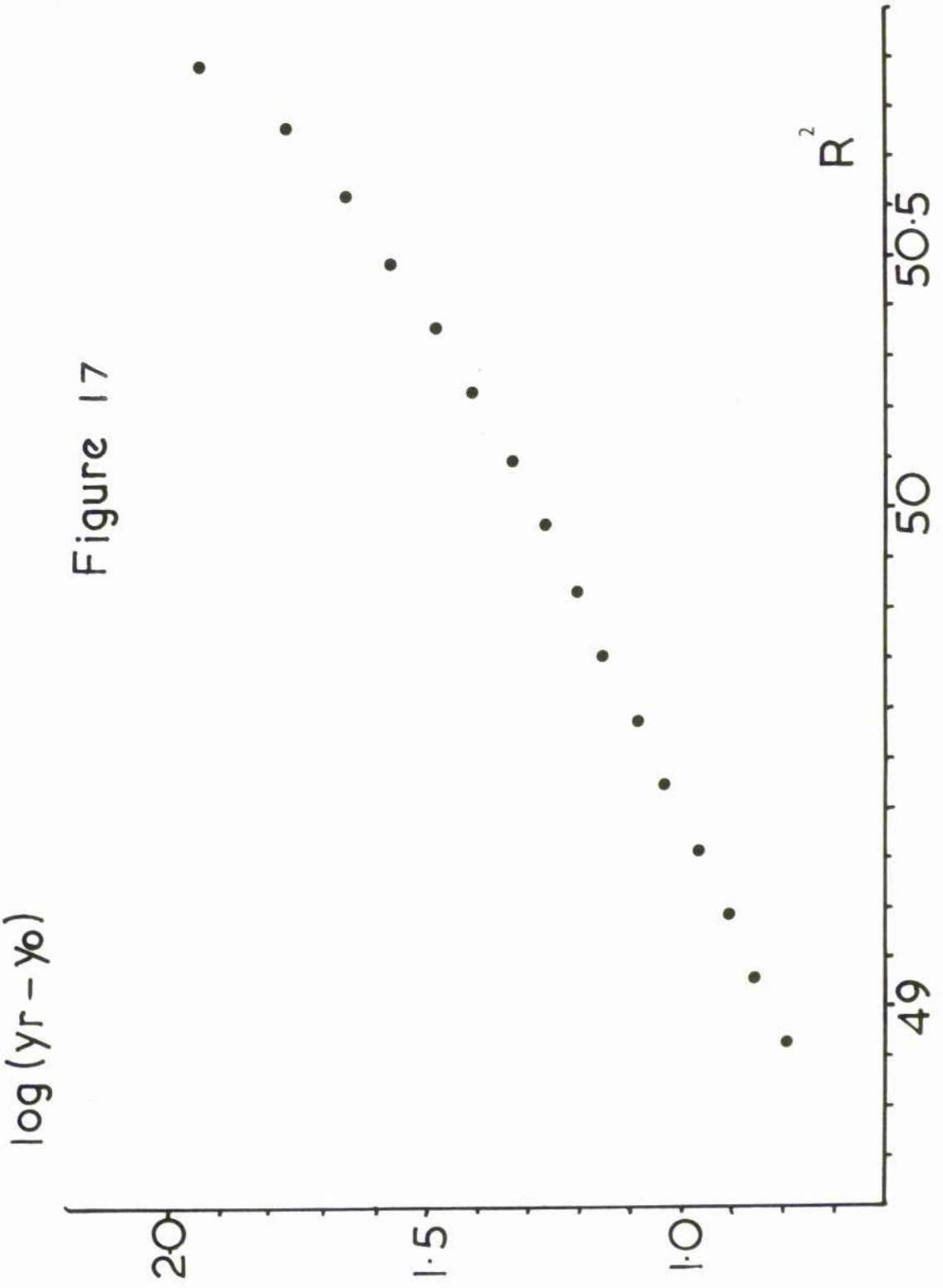


Figure 17

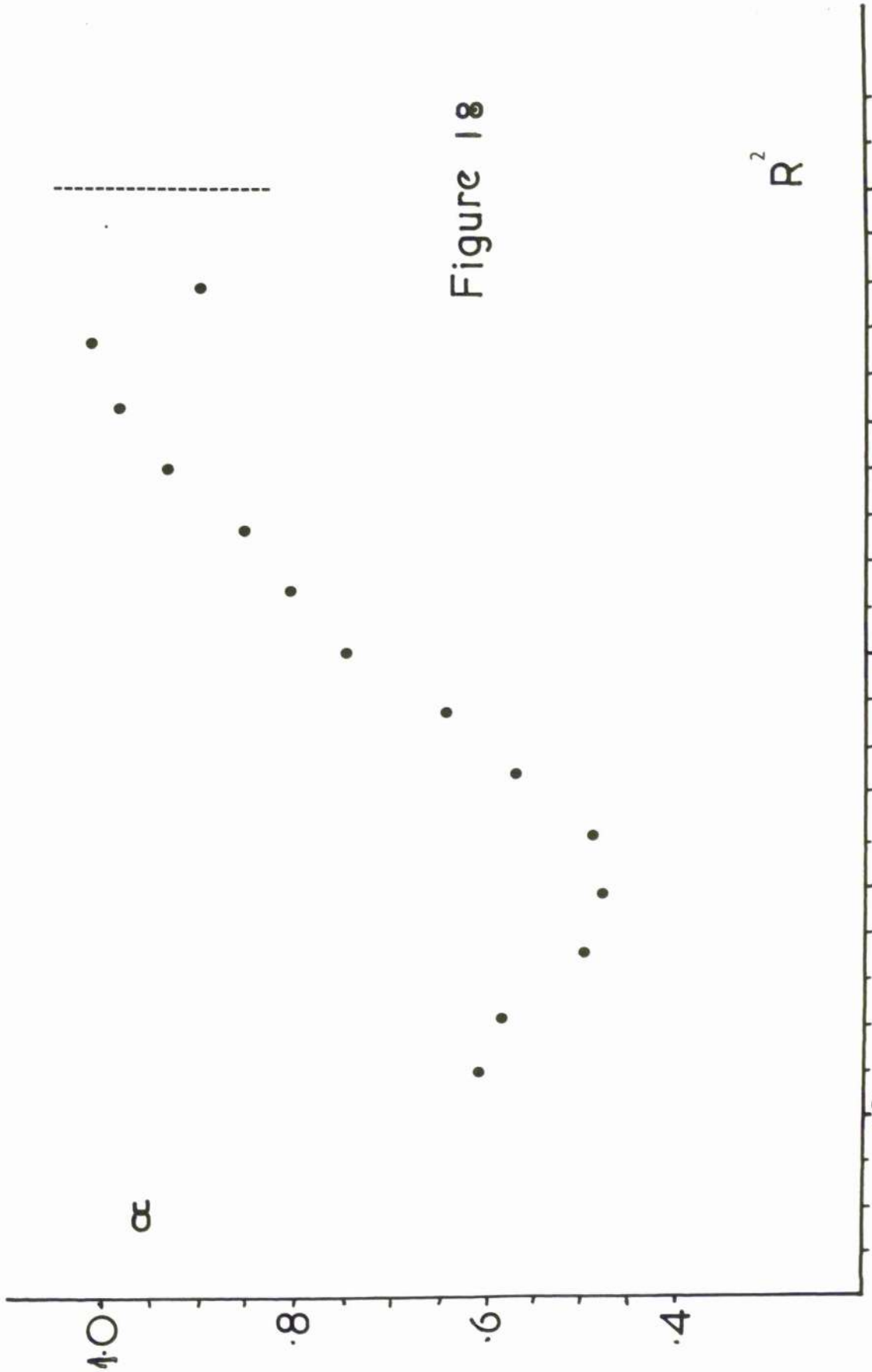


Figure 18

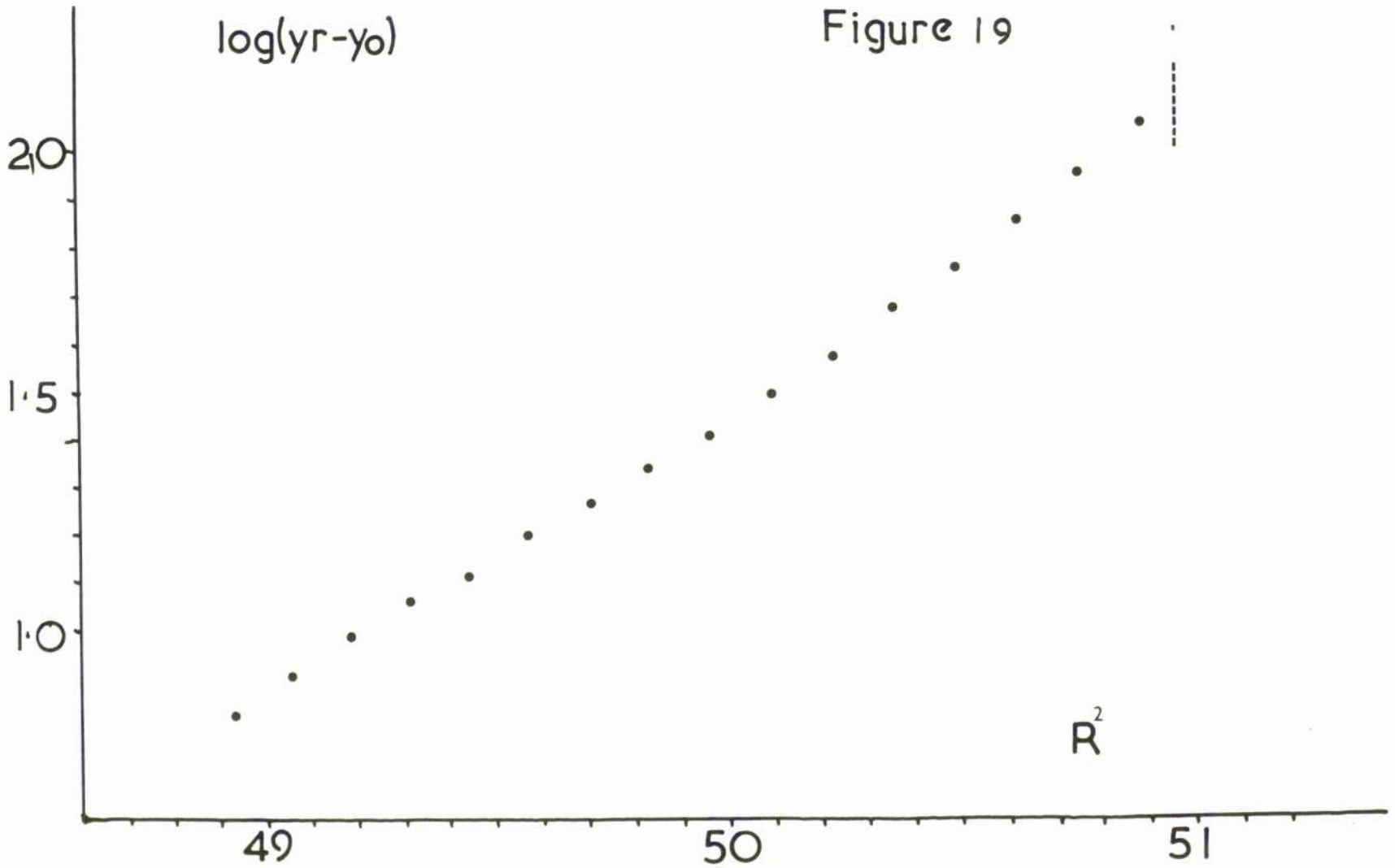


Figure 19

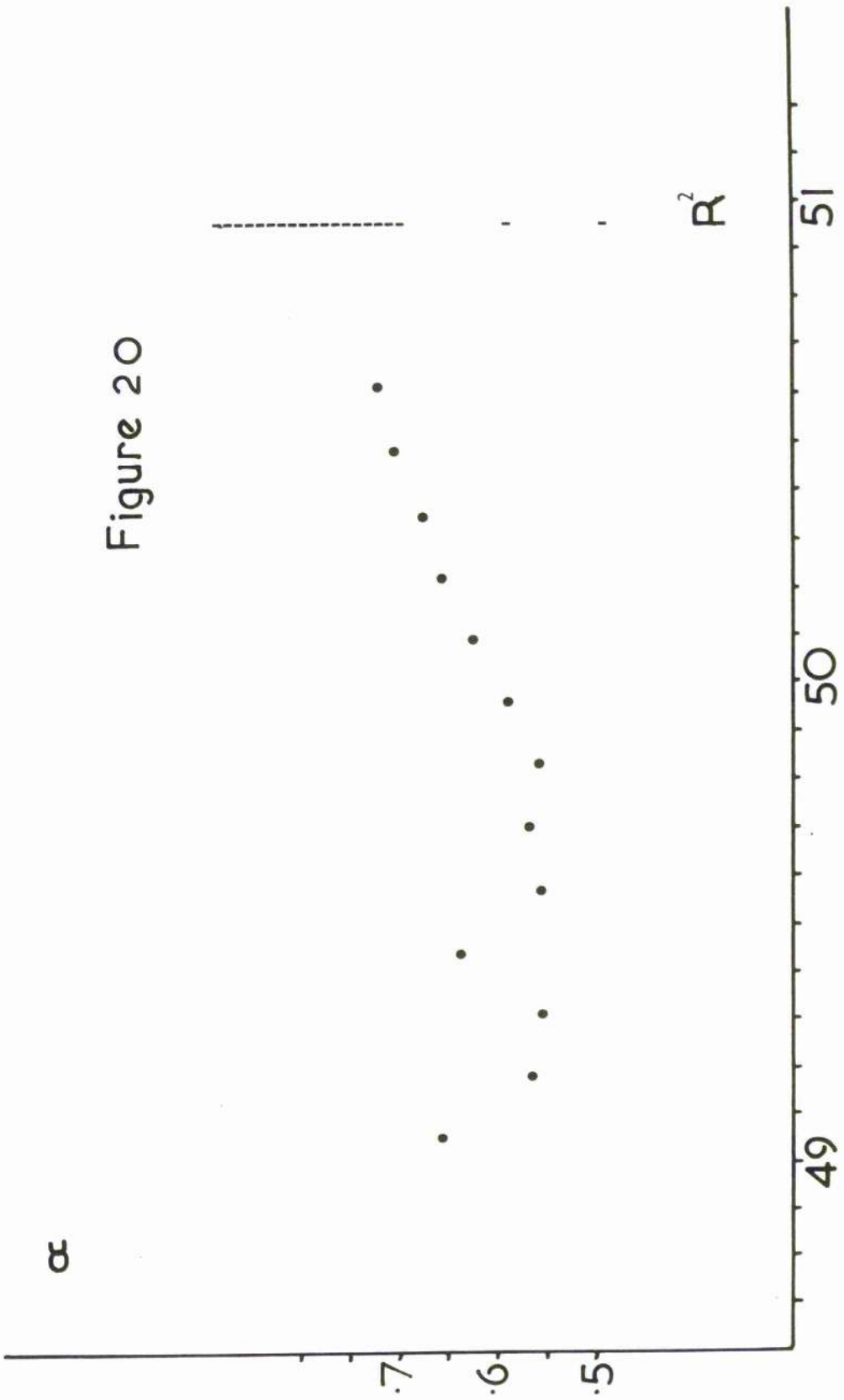


Figure 20

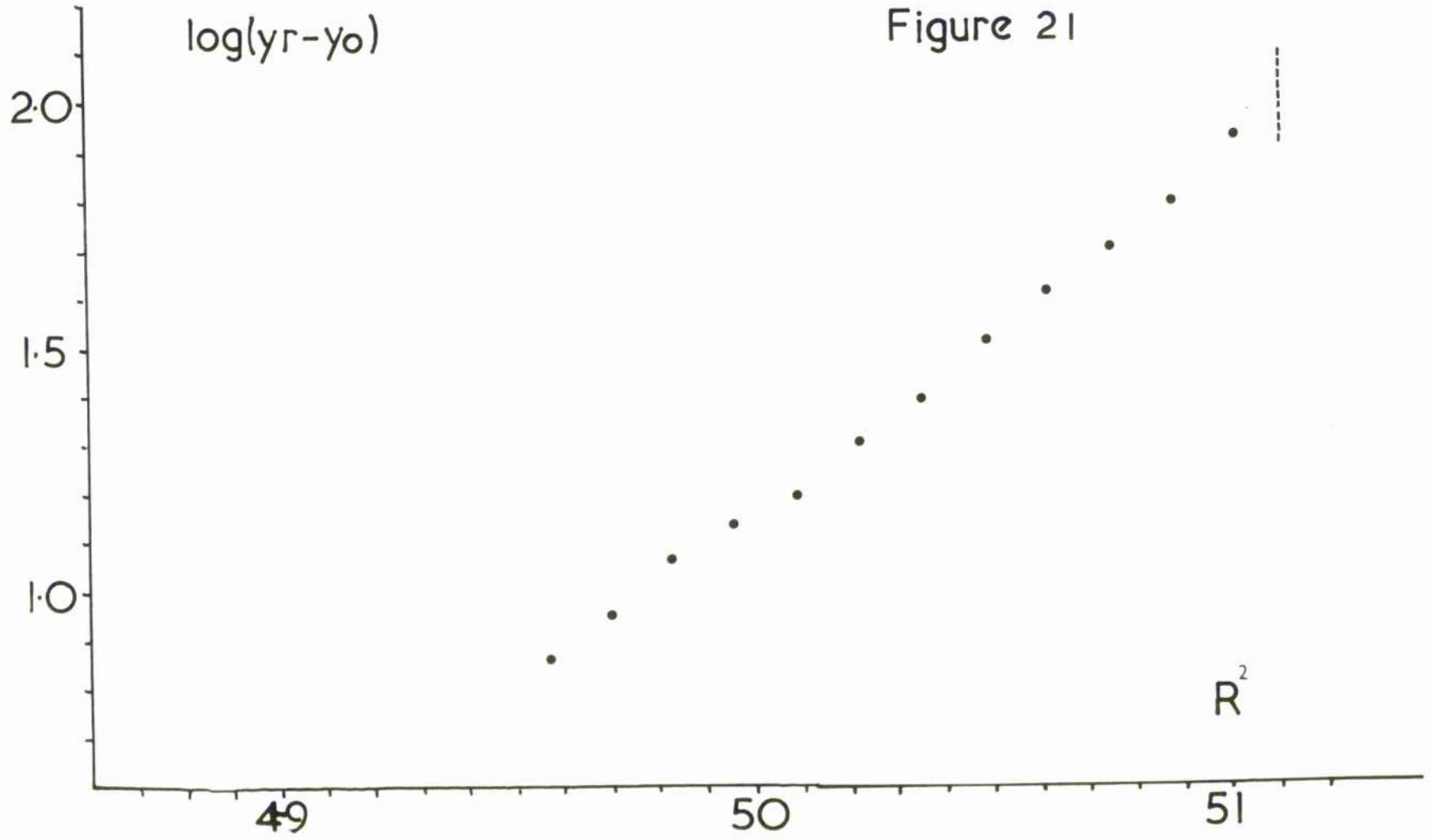


Figure 21

Figure 22

Figures 23 and 24

Sample: Fraction 2 ; retained by 200 - 350 Å cellulose acetate filter. Not retained by 500 Å cellulose nitrate filter. About 1.5% of treated PG.

concentration	=	*0.33 mg ml^{-1} in 2% KCl*
speed	=	*27,690*
$\bar{v}^*$	=	*0.660*
α_ω*(base)*	=	*0.681*
$\bar{M}z$*(base)*	=	*27,290*
α_ω *from Fig.23*	=	*0.679*
corresponding $\bar{M}w$	=	*27,200*

There is no evidence of a 'monomer' species so α_ω*(meniscus) has not been calculated.*

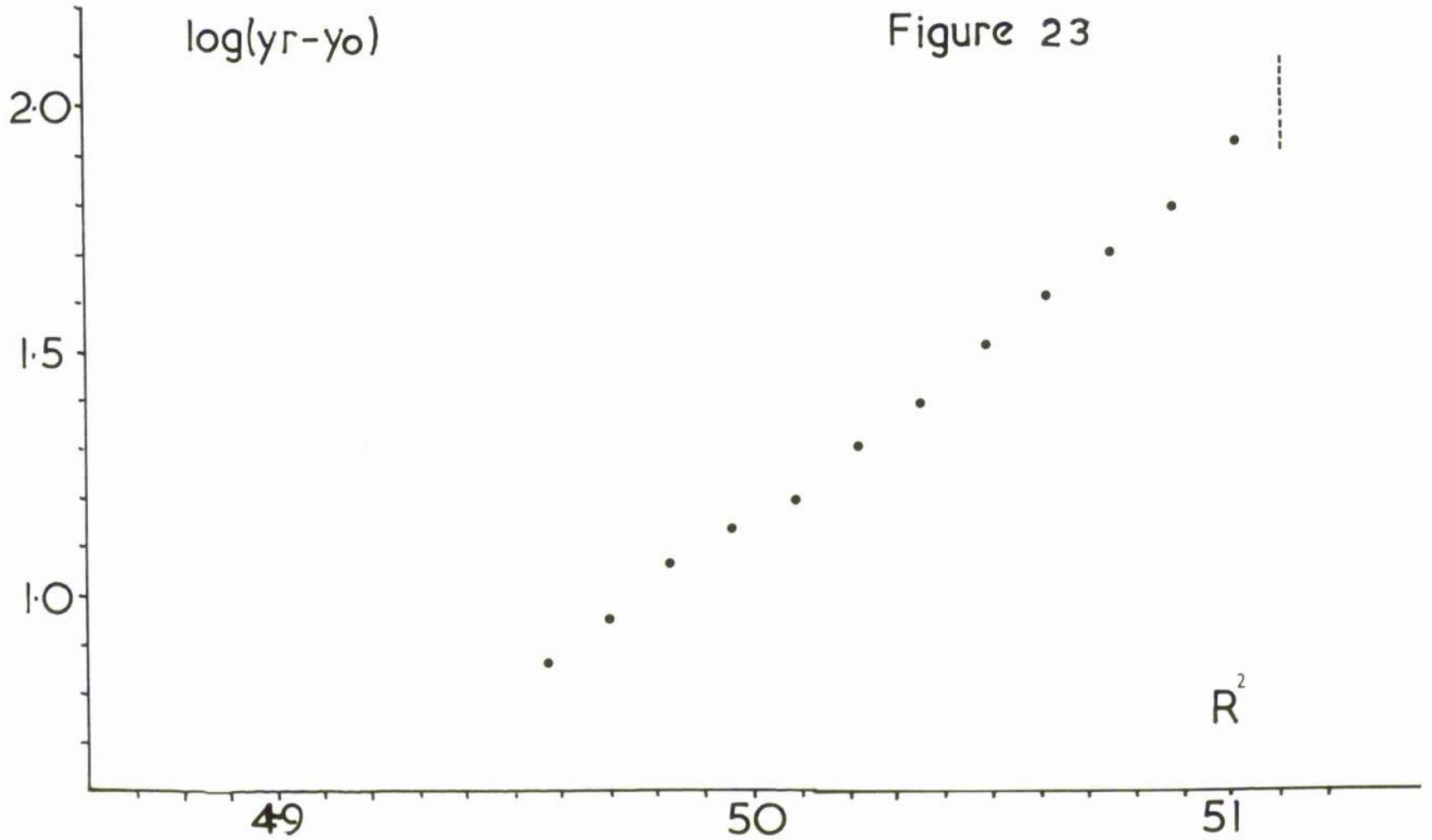


Figure 23

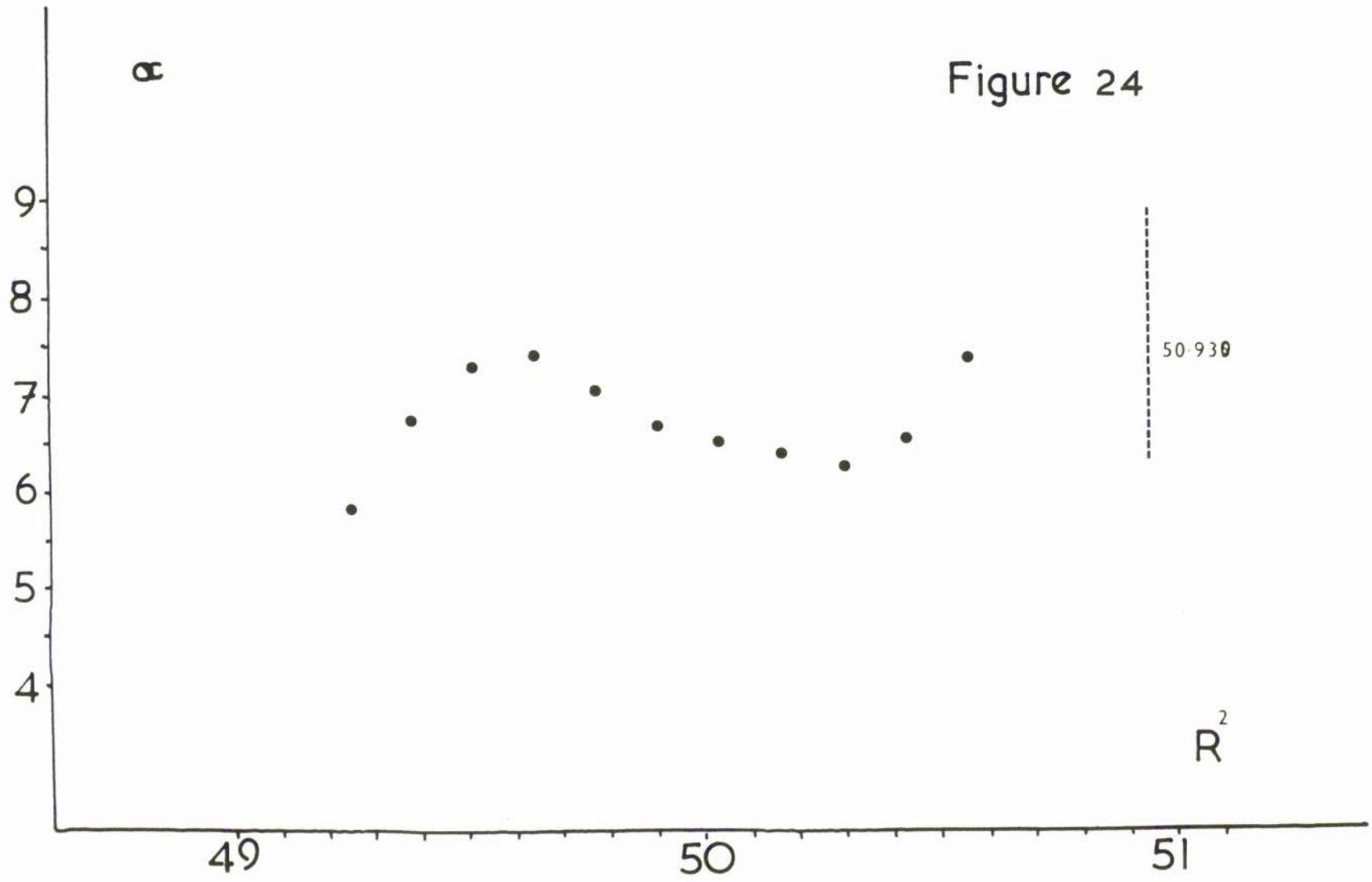


Figure 24

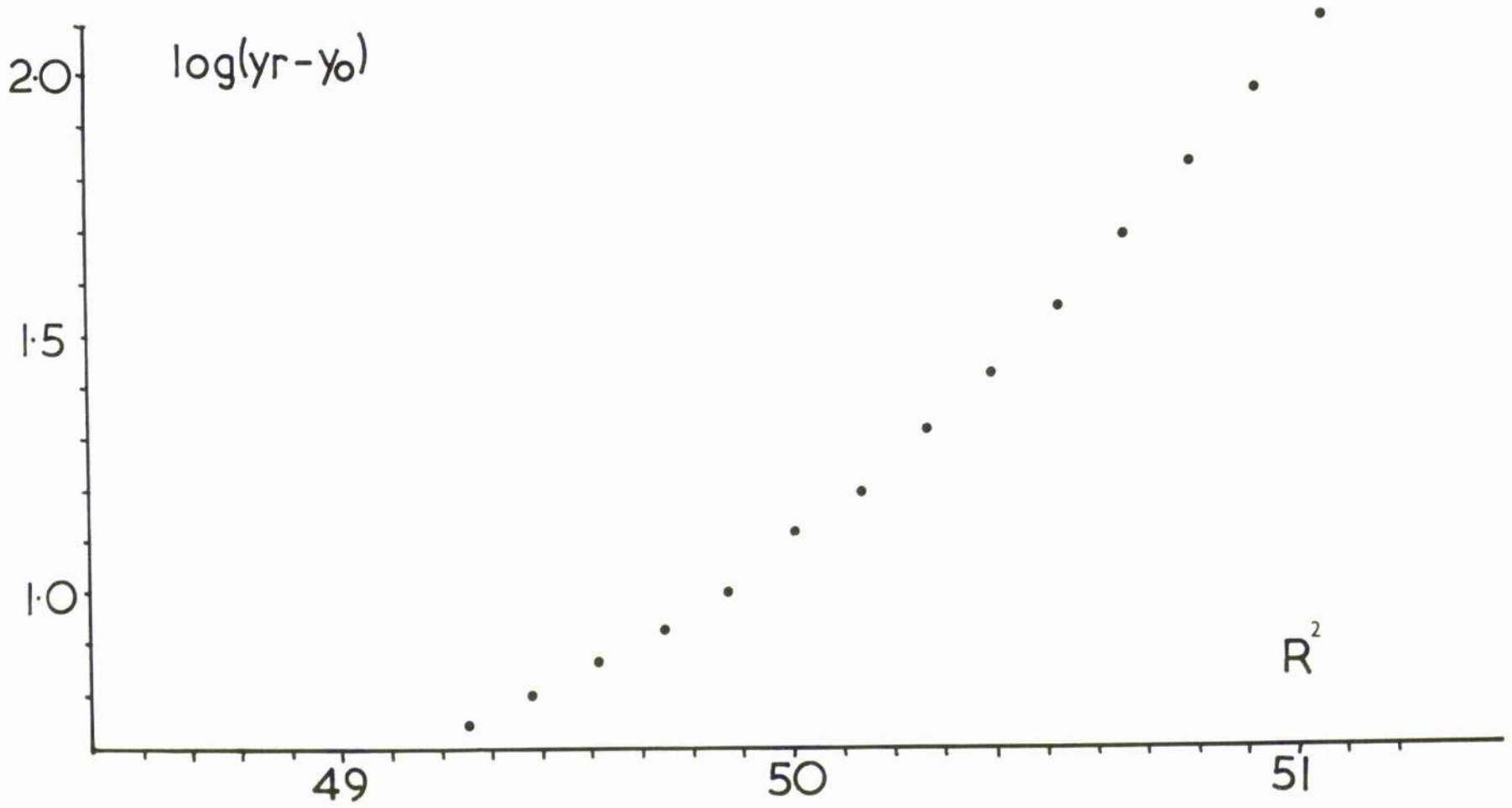
Figures 25 and 26

Sample: Fraction 3 ; retained by 500 Å filter (cellulose nitrate)
Not retainea by 1000 Å filter.
About 1% of treated PG.

concentration	=	*0.30 mg ml^{-1} in 2% KCl*
speed	=	*24,640*
$\bar{v}^*$	=	*0.653 (estimated)*
α_ω*(base)*	=	*0.466*
$\bar{M}z$*(base)*	=	*23,100*
α_ω*(meniscus)*	=	*0.282*
$\bar{M}\omega$*(meniscus)*	=	*14,000*

The evident high M.W. species have been ignored in the extrapolat

Figure 25



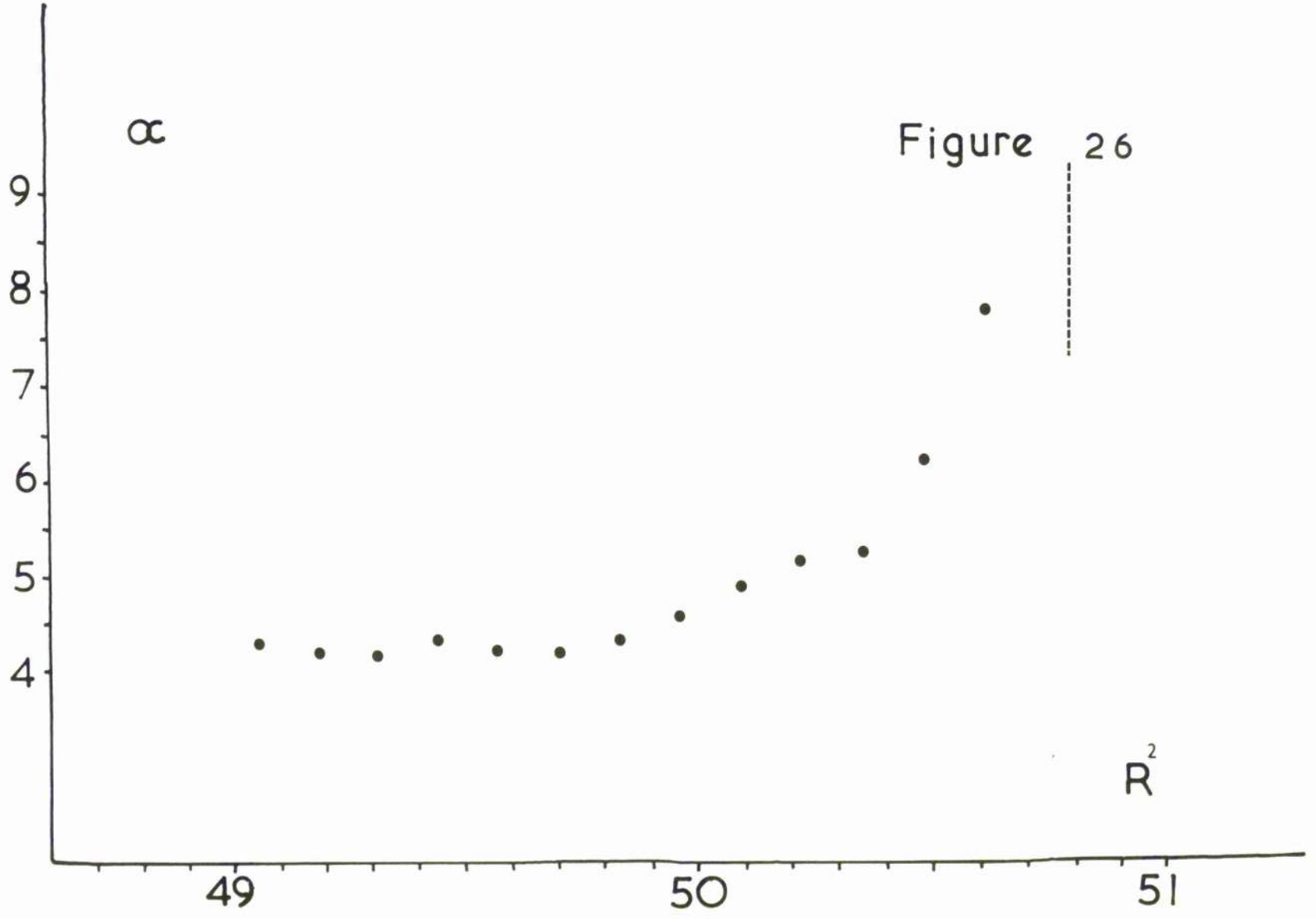


Figure 26

Figures 27 and 28

Sample: Fraction 4 ; trace amount retained by 1000 Å filter
Not retained by 2000 Å filter. (Cellulose nit.

concentration = *about 0.3 mg* ml^{-1} *in 2% KCl*

speed = *27,690*

$\bar{v}^*$ = *0.650 (estimated)*

α_ω*(base)* = *0.533*

$\overline{M}\omega$*(base)* = *21,040*

lowest values for α_ω *are 0.38/0.40*

corresponding $\overline{M}\omega$ = *15,790/15,000*

Polydispersity prevents estimate of 'monomer'.

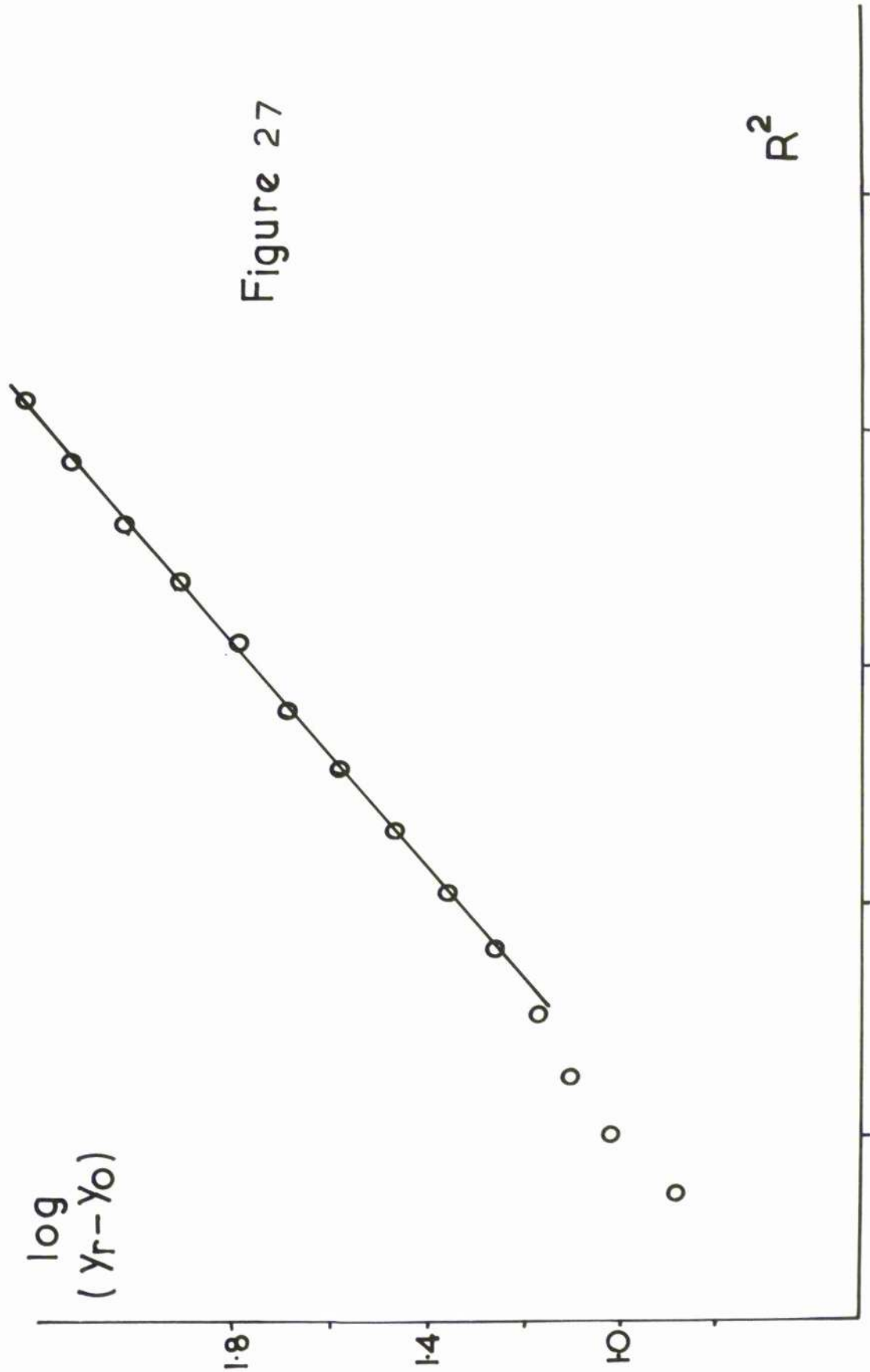


Figure 27

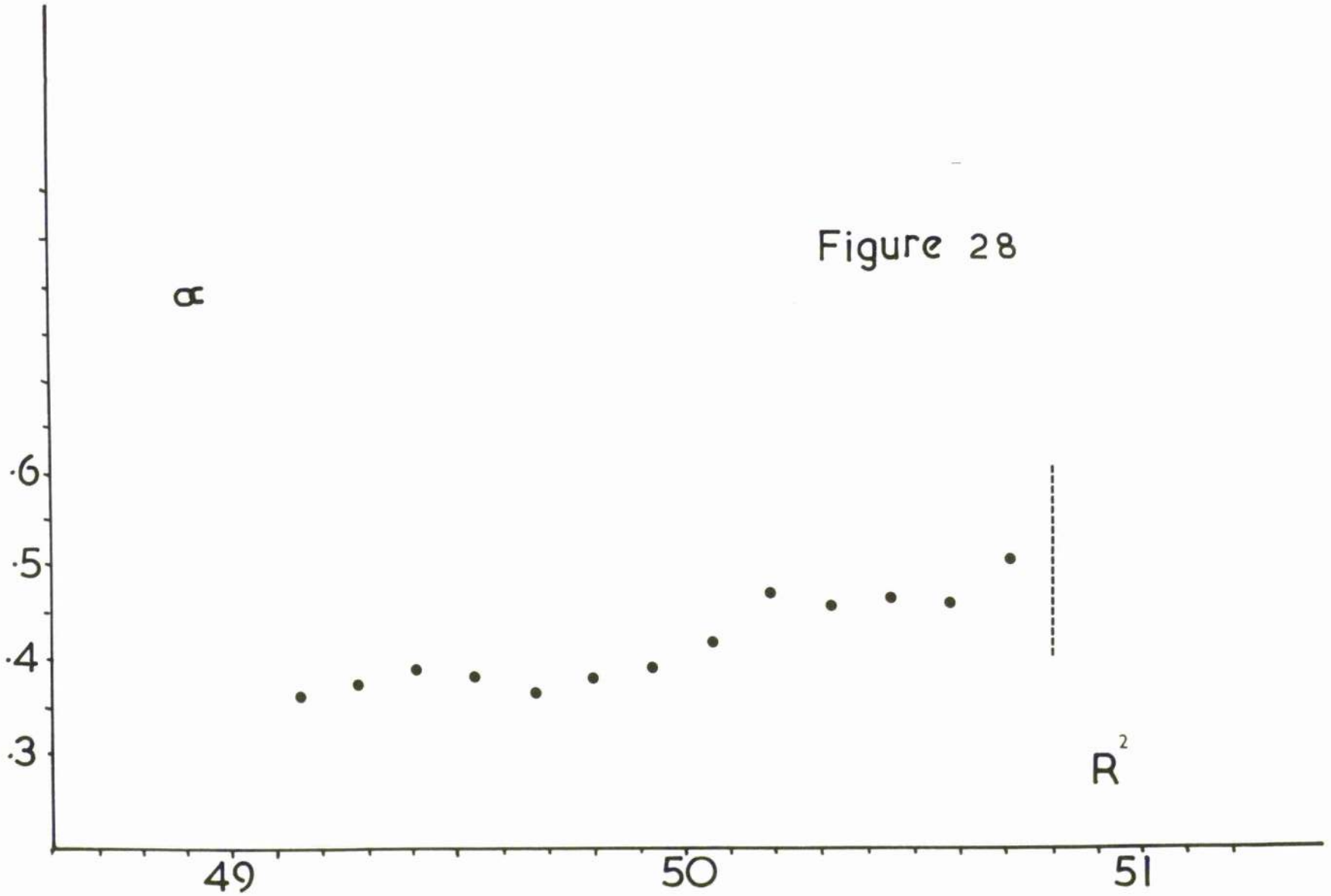


Figure 28

FIGURE 29

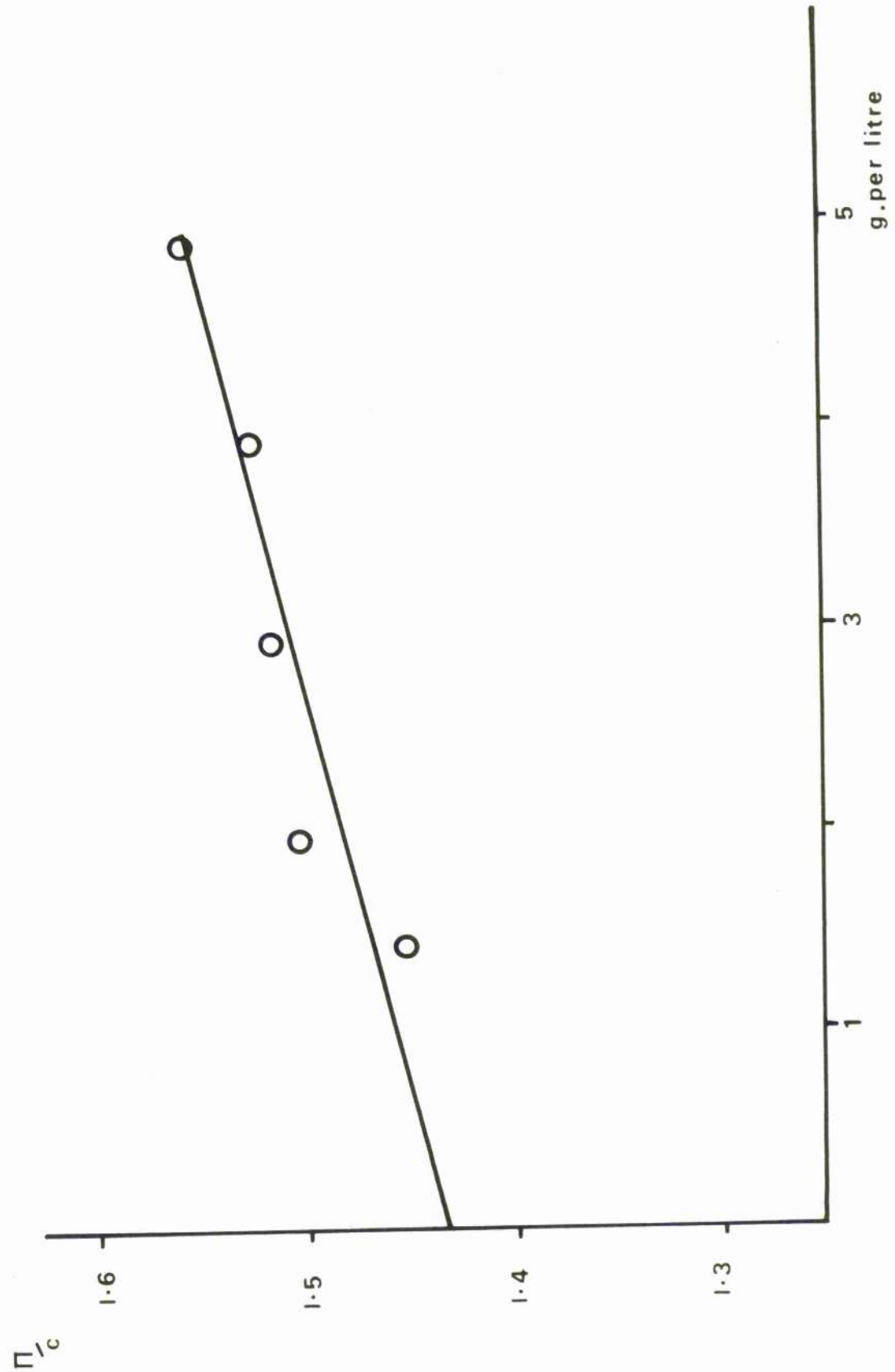
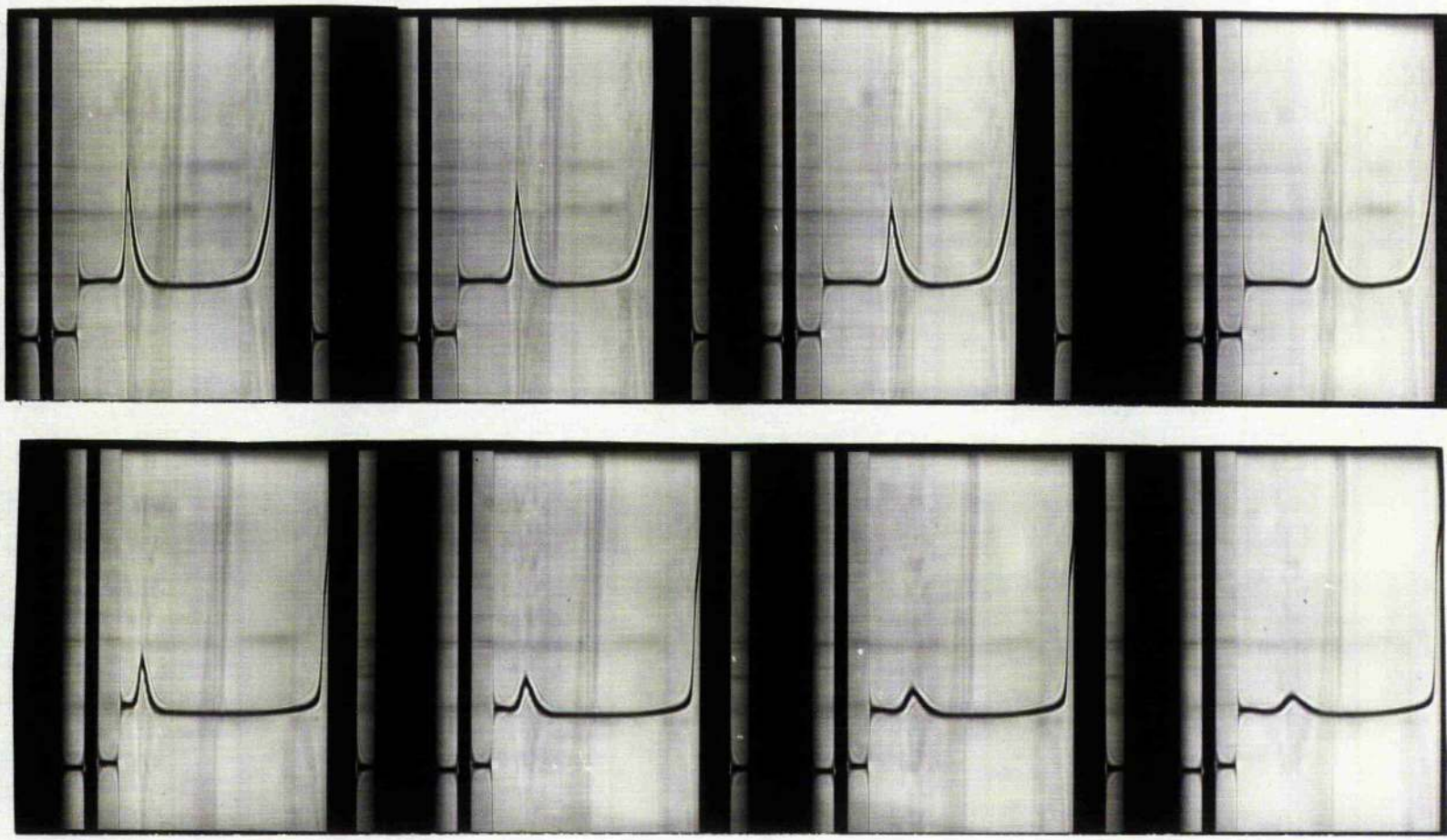


Figure 30

Schlieren photographs for fraction 5 at two concentrations. It is clear that 1. The peaks are asymmetric and 2. They break down rapidly. The corresponding photographs for PG are not shown.

FIGURE 30



The Schlieren photographs were taken at 20 minutes. Speed was 59,780 rpm. The sample is fraction 5. (2% KCl) and concentrations were 6.6 mg ml^{-1} (upper) and 0.33 mg ml^{-1} (lower).

S_{20} at 6.8 mg ml^{-1} = 3.85 ± 0.1

S_{20} at 3.5 mg ml^{-1} = 7.45 ± 0.1

For PG : S_{20} at 6.6 mg ml^{-1} = 5.56 ± 0.4

S_{20} at 3.3 mg ml^{-1} = 11.01 ± 0.7

Amino acid analysis

Analyses were carried out on PG, F1, F2, F3, and F5. The results are shown in Table 2. Protein contents were calculated as % of total 'ion exchange recoverable' material. Since neutral sugars form some 5% of PG (Luscombe and Phelps, 1967) the results will be in error by amounts up to this figure.

	PG	F1	F2	F3	F5
Protein content %	15.78	1.70	5.98	2.50	7.41
Ash %	30		15		24
Keratan/ chondroitin ratio	1:12.1	-	1:11.0	1:36.6	1:13.6

Table 2

Amino acid analysis of PG and derived fractions

Results are expressed in the form: microgram % of total amino acids.

	PG	F1	F2	F3	F5
asp	9.91	6.54	7.48	7.07	11.25
thr	5.57	5.40	6.22	5.94	3.36
ser	8.84	11.31	10.63	9.23	10.93
glu	16.55	15.65	17.04	15.18	19.02
pro	9.29	9.70	10.40	8.36	9.87
gly	6.92	7.32	5.49	10.16	4.88
ala	4.67	10.67	4.59	0.55	4.02
val	5.72	5.01	5.08	6.79	5.97
met	0.45	-	0.39	0.96	0.25
ile	3.78	3.65	3.36	3.95	3.76
leu	8.29	9.34	6.50	8.47	8.46
tyr	1.89	1.64	4.09	6.56	1.80
phe	5.51	4.64	6.69	3.14	3.73
orn	-	-	1.92	2.72	0.45
lys	3.63	2.51	2.88	2.48	3.37
his	2.47	1.38	3.04	3.42	2.85
arg	6.53	5.22	4.18	4.31	6.03

Cysteine and hydroxy lysine were completely absent from all samples.

Analysis of F1 was carried out on the Technicon Analyser which does not have the sensitivity of the Locarte Analyser and could not detect the met

DISCUSSION

The molecular weight of 4.4 million obtained by light scattering is in reasonable agreement with other values quoted for PG extracted by the method of Malawista and Schubert. The model proposed by Mathews and Lozaityte (1958) had a molecular weight of 4 million and a radius of gyration of 1850 Å while Luscombe and Phelps (1967) reported values of 5.8 million and 1,390 Å. The experimentally derived value for R_G of 2,300 Å seems acceptable in the light of the undoubted polydispersity of PG. Particularly in the high molecular weight range, light scattering is limited in this type of study by its extreme sensitivity to just a few % of any high M.W. material. Apart from its use the characterization of native-extracted PG, the technique was introduced into this work in anticipation of the problem associated with physical studies on asymmetric molecules with molecular weights greater than about $5 - 6 \cdot 10^5$.

The molecular weight analyses on the fractions isolated by ultrafiltration basically show that all fractions, with the exception of F2, are polydisperse. Since the primary aim of this study has been a feasibility exercise, the possible structural significance of these fractions will not be

discussed in any detail here. However one or two points require comment. Since Serafini-Fracassini et al. (1967) and Serafini-Fracassini (1968) have shown that acid/acetone treatment of PG does not apparently increase the number of N-terminal amino acids, it would seem that scission of peptide bonds cannot be invoked to explain the formation of subunits. This implies that the protein core is not continuous in the 1 - 5 million M.W. PG species, and this is supported to some degree by the close agreement in amino acid analyses between the various fractions and native PG. The similarity between F1 and PG is particularly surprising for a molecule in the range 12 - 18 . 10^3, as is also the unexplained variation in alanine and tyrosine. There is some similarity in these results with the fraction described by Partridge (1966) of molecular weight 240,000 and protein content 7%.

With reference to results previously described in the literature for acid/acetone treated PG, it should be stressed at this point that the total % of fractions (by weight) which passed through a 2,000 Å filter comprised less than 10% of the total material so these fractions cannot be regarded as being representative of the entire sample. Since the treated PG had a strong tendency to aggregate as shown in the Schlierentrace of F5 (Figure 30)(A lot of material is evident at the base of the column), it would seem that

ionic strength and pH are critical factors in the behaviour of the treated material. (The separation by Serafini-Fracassini et al. (1971a) was carried out by gel chromatography in 1 M potassium acetate).

As far as the fractionation is concerned, it appears that the technique is of limited use. There is only a slight correlation between filter pore size and molecular weight. One possible explanation is a charge effect at the surface of the filter. On the other hand a possible source of confusion could have arisen from a phenomenon described i a recent (frightening) paper by Di Prisco and Strecker (196 These authors have reported that 12 hour dialysis or ultrafi ration in vacuo caused as much as 20% hydrolysis of glutama dehydrogenase. The ultrafiltration used in this work was no carried out in vacuo but extended dialysis was used in both de-salting and preparation of solutions for ultracentrifuga Roden (1970) has emphasised that glycosidic bonds are easil broken a) by thermal cleavage, b) by strongly reducing substances such as ascorbic acid and c) mechanical depolymerisation. The cleavage of glycosidic linkages during isolation procedures may thus occur to a larger extent than is generally realised. If peptide bonds can be as easily broken as has been suggested by Di Prisco and Strecker then glycosidic links must fare even worse. Such an explanation might well account for the observed polydispersity in most c

the fractions. This seems even more likely in the light of an imaginative comparison between molecules encountering a dialysis membrane and those undergoing high pressure filtration through similar material. It seems unlikely that acid/acetone treatment causes the polydispersity in that the number of reducing groups (related to glucose as a standard) in the tre ted PG is not significantly different from that i the untreated material. The results showed that both samples contained 11 $\pm$ 3Y equivalents of glucose per mg of m terial. The method used was a modification of the prussian blue reac suggested by Park and Johnson (1949). However it was found t the determination was sensitive to the presence of salts and it was necessary to dialyze the samples against water before estimation. Oligosaccharides could easily be lost in this wa so these are not presented as completely reliable results.

The question of breakdown during dialysis occurs again in the interpretation of the results on F1 by osmometry. The $\overline{M}n$ of 20,000 is clearly at variance with the maximum $\overline{M}z$ valu of 18,000 obtained from ultracentrifugation. One possibility is that the correction for $\overline{v}$ has not been sufficiently preci As mentioned previously, associated ion corrections can only of limited accuracy. Another source of error could have aris in the osmometry from loss of small molecular weight fragmen through the membrane. There was slight evidence for this in the equilibrium times for each concentration were about 20 t 30 minutes which is a little longer than usual. Further

experiments were not attempted since previously, considerable difficulty had been experienced with membranes other than the B-19. Certainly, selection of membranes is a critical factor in investigations below 20,000 M.W.

Sedimentation velocity experiments on F5 (Figure 30) sho that it was also polydisperse. This is particuarly evident in the low concentration run, where the peak is seen to disinteg rapidly. The S values seem to indic te that the molecular wei less than that of PG (assuming their shapes are comparable). Due to doubt concerning the validity of S value extrapolation the S^{o}_{20} values are not given. The nonlinear relation (even wi a 1/S plot) between the concentration and sedimentation coefficient indicated the desirability of investigating sedimentation behaviour below 1 mg ml^{-1}. This is not feasible using Schlieren optics. Although sedimentation coefficients are probably the most frequently quoted physical parameter of macromolecules , it is surprising how infrequently the validi of the zero concentration extrapolation is questioned.

Finally there is one seemingly minor problem that has no so far been mentioned. This is the determination of concentra This is not of direct importance in the sedimentation equilibrium studies (provided the meniscus depletion techniqu is used) but it is critical in the determination of $\bar{v}$ and $\bar{v}^{*}$ This is part of the problem of determining T. Since dry weigh are fequired the inaccuracy will be large unless there is sufficient material to make the % error small - yet another

reason why techniques enabling preparation of relatively large amounts of material must be developed if meaningful physical parameters are to be obtained.

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